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V. B. WIGGLESWORTH and J. A. RAMSAY

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HORMONAL CONTROL OF SEXUAL MATURITY  
IN *OCTOPUS*

BY M. J. WELLS AND J. WELLS

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(Received 3 June 1958)

(With Plates 1-2)

## INTRODUCTION

During the last few years an intensive programme of experiments designed to correlate structure with function within the brain of cephalopods has been carried out by two groups of workers. Boycott and Young have studied the effect of brain lesions upon visual learning (for references see Boycott & Young, 1955; Young, 1958) and the present authors (Wells & Wells, 1957-8) have studied the effect of lesions on tactile responses. In both series of experiments animals have been found with enlarged gonads, and a preliminary note in which three cases (two males, one female) are cited as occurring after optic tract section has been published by Boycott & Young (1956). The much more frequent occurrence of enlarged gonads in experiments on tactile responses, in which blinded animals were used, led us to begin a systematic investigation of the causes of this phenomenon. We started collecting records of the condition of the gonads in our experimental animals in August 1954 and by the end of August 1957 had accumulated data from 487 individual octopuses, including samples from every month of the year except January. The present account is an analysis of these data and shows that maturation of the gonad is determined by a secretion from the optic glands; production of this secretion is regulated by a centre in the supraoesophageal brain mass and ultimately by light.

## MATERIAL

Octopuses of from 200 to 1000 g., probably aged from 6 to 18 months (see p. 6), were collected from the Bay of Naples. These animals were killed by cutting out the brain and weighed immediately after death, weights being recorded to the nearest 10 g. Gonads were dissected out together with their ducts and weighed to the nearest 0.1 g. The majority of these records were from animals treated in various ways in connexion with work on learning in *Octopus*, in particular with work on the animal's tactile system. For the latter experiments animals were subjected to operations in which they were blinded by section of the optic nerves and/or had parts of their brains removed. Descriptions of the operational techniques involved are given in Wells & Wells (1956, 1957*a*). After such operations animals were used in training experiments of variable duration before being killed; brain lesions were

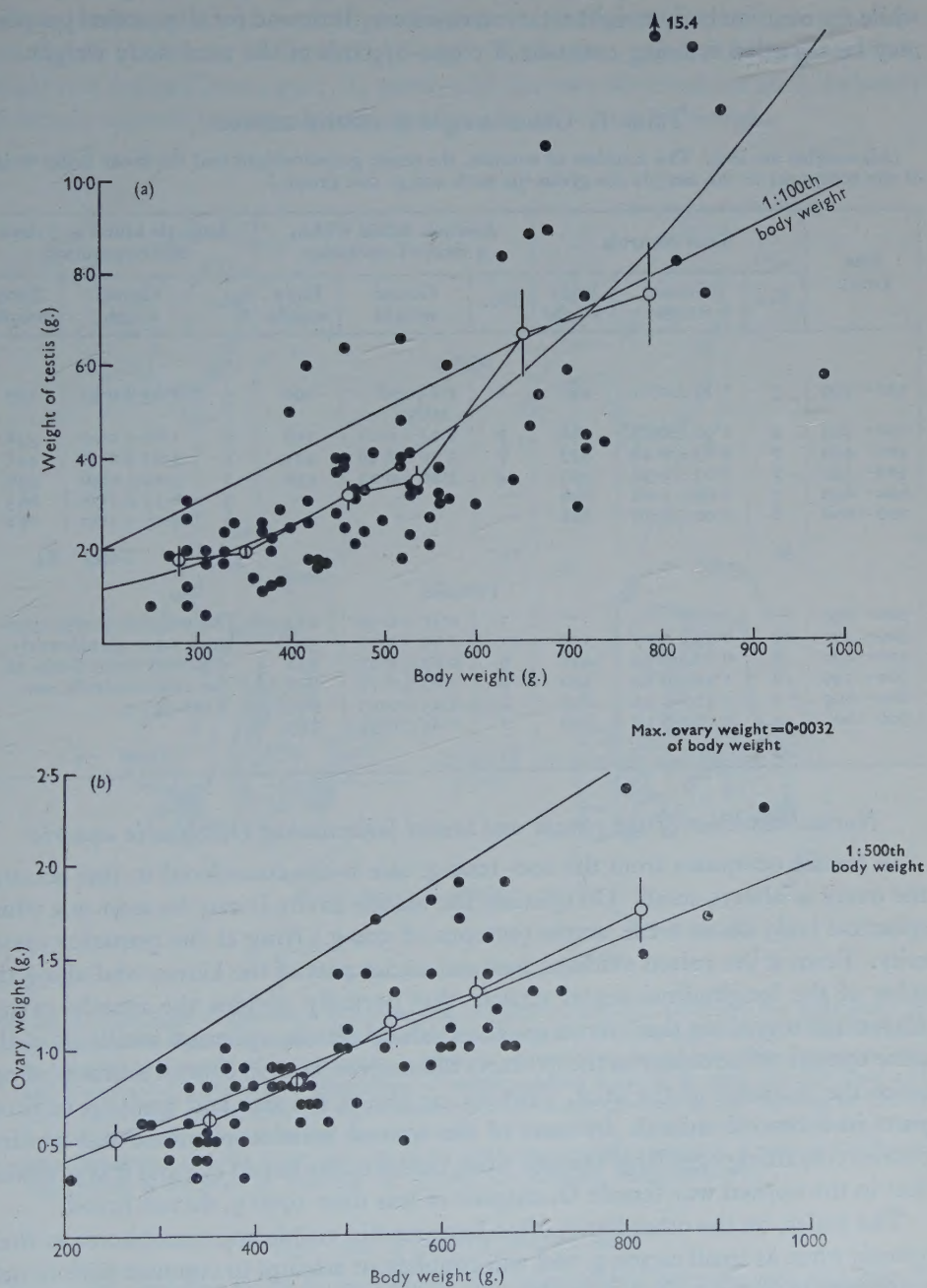
then checked from serial sections. At least two variables must therefore be taken into account when considering the effect of central brain lesions and the effect of optic nerve section upon the state of the gonads—the nature of the lesion made, and the time elapsed between operation and killing the animal. The possibility of a third variable, seasonal variation in the state of the gonads, was also considered but although records were collected in every month of the year (except January) no obvious seasonal differences were found. It is possible that with more data small seasonal variations would prove detectable, but this is unimportant to the present analysis since enlargement of the ovary, upon which most of the experimental results are based, is unmistakable when it happens and has not been observed to occur spontaneously at any time of the year in animals within the size range (200–1000 g.) considered, although such animals will copulate (see page 4). Males with spermatophores in their testicular ducts have, on the other hand, been observed at all times of year.

The experimental animals were kept separated from one another, whenever possible in individual tanks. In some experiments animals were kept in large tanks divided into stalls by partitions. This was not entirely satisfactory as a means of separating the animals which were sometimes able to touch one another and even copulate (see Pl. 2*a*) through gaps left for sea-water circulation around the edges of the partitions.

In the text, tables and figures individual animals are referred to by the number with which they were identified in our original protocols. The prefix to this number *A*, *B*, *C* or *D* gives the year in which the animal was used, being 1954–7 respectively. Other animals are identified by a three-letter reference; these are octopuses used in visual training experiments by Boycott & Young, who kindly allowed us to collect data on gonad and body weights from their animals and also placed at our disposal serial sections of the brains of those that we wished to examine because they had enlarged gonads. The method of mapping brain lesions used in this account is explained on p. 8.

#### *Gonad weight in control animals*

Because octopuses are expensive to buy in Naples, a comparatively small number, thirty-two males and thirty-five females, were killed without first being used in experiments involving surgical interference with the nervous system. To these true controls we can add a further fifty-two males and thirty-seven female animals that died or were killed within a few days of operation, having gonad weights that did not differ significantly from those of the true controls (see Table 1). In the case of males this means animals killed within 7 days of operation; in females, where the gonad enlargement as a result of operation is much more spectacular, significant differences in gonad weight are found as little as 4 or 5 days after operation, and the limit of the period during which it seems justifiable to include records from operated animals amongst the controls is reduced to 3 days. The gonad weights from the total of eighty-four male and seventy-two female controls are summarized in Text-fig. 1. The weight of the testis is more variable and nearly always greater than that of the ovary from animals of the same size; moreover the testis weight



Text-fig. 1. Weight of the gonad plotted against body weight in control males (a) and females (b). Mean and standard error is given for each 100 g. size group (the last group includes all animals between 700 and 1000 g.). Where two individual records would be superimposed one is displaced to the right.

shows a marked increase relative to the body weight over the range considered while the ovary to body weight ratio increases very little and for all practical purposes may be regarded as being constant at 0.002–1/500th of the total body weight.

Table 1. *Gonad weight in control animals*

(All weights are in g. The number of animals, the mean gonad weight and the mean body weight of the octopuses in the sample are given for each 100 g. size group.)

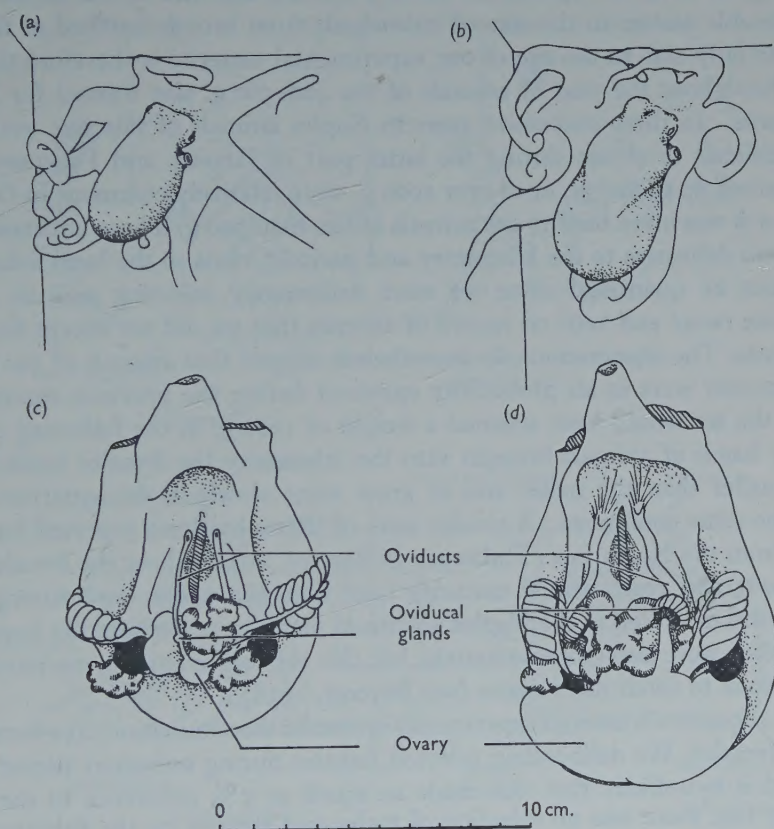
Size group	True controls			Animals killed within 3 days of operation			Animals killed 4–7 days after operation		
	No.	Gonad weight	Body weight	No.	Gonad weight	Body weight	No.	Gonad weight	Body weight
Males									
200–299	3	1.17 ± 0.61	280	1	1.2 (one only)	290	3	1.93 ± 0.54	283
300–399	4	1.90 ± 0.35	368	7	2.04 ± 0.20	356	5	1.66 ± 0.27	338
400–499	7	2.83 ± 0.42	437	7	3.50 ± 0.48	441	9	3.23 ± 0.46	441
500–599	7	3.03 ± 0.34	529	4	3.20 ± 0.23	538	7	4.24 ± 0.56	546
600–699	5	6.80 ± 1.08	646	—	—	—	3	6.57 ± 1.78	663
700–1000	6	7.00 ± 1.16	828	—	—	—	6	8.17 ± 1.82	752
	32			19			33	Total	84
Females									
200–299	—	—	—	3	0.50 ± 0.09	253	The weights in this category were significantly different from those of the true controls, see Text-fig. 9.		
300–399	10	0.55 ± 0.05	359	11	0.62 ± 0.07	348			
400–499	8	0.81 ± 0.04	448	9	0.83 ± 0.07	446			
500–599	6	1.22 ± 0.14	540	6	1.07 ± 0.16	550			
600–699	7	1.17 ± 0.12	635	6	1.45 ± 0.13	643			
700–1000	4	1.70 ± 0.19	820	2	1.85 ± 0.39	765			
	35			37			Total 72		

*Normal condition of the gonads and sexual behaviour of Octopus in aquaria*

In female octopuses from the 200–1000 g. size range considered in this account the ovary is always small. On opening the mantle cavity it may be seen as a white spherical body about 1 cm. across (octopus of 500 g.) lying at the posterior extremity. From it the paired oviducts pass out under part of the kidney and along the sides of the longitudinal septal muscle that partially divides the mantle cavity. About half way along these ducts are the oviducal glands, spherical swellings of the same opaque white colour as the oviducts themselves, and, in control animals, about twice the diameter of the duct. Text-fig. 2c shows the size and position of these parts in a control animal. In none of the control females examined did we find ovaries containing eggs large enough to be visible to the naked eye and it is probable that in the normal way female *O. vulgaris* of less than 1000 g. do not breed.

The males, on the other hand, often have readily visible spermatophores in their gonads when as small as 300 g. and will copulate or attempt to copulate with female octopuses of similar size. A male octopus will also attempt to insert its hectocotylyzed third right arm into the mantle cavity of other males. Both males and females resist this intrusion but the resistance is by no means as vigorous as Pelseneer (1935) suggests—'La femelle se défendant des bras et du bec pendant

toute la durée de l'acte qui gêne sa respiration'\*—and situations such as that shown in Pl. 2a (where the female could readily have moved away) are not uncommon in aquaria. As in the case of *Sepia* (Bott, 1938; Tinbergen, 1939), *Sepiolo* (Racovitza, 1894b) and *Loligo* (Drew, 1911, *L. pealii*, and our own observations on *L. vulgaris*) the female appears to play a purely passive role in mating behaviour.



Text-fig. 2. Condition of the ovary in normal and in operated animals. (a) shows the body shape of an unoperated female octopus, and (b) that of an animal of similar size 2 days before it began to lay eggs as a result of an operation in which a central brain lesion was made (octopus C 27, see Table 2). (c) and (d) are dissections of corresponding animals; in each case the head and arms have been cut off and the septal muscle to the mantle severed so that this can be folded back to show the contents of the mantle cavity. Octopus D 12, shown in (d), was killed 37 days after operation and the ovary and its ducts were very greatly enlarged; a map of the lesion responsible is included in Text-fig. 7c. (From Kodachrome transparencies.)

#### Age, rate of growth and sex ratio

Very little appears to be known about the relationship between age and size in *Octopus*. The animals grow very rapidly indeed in aquaria; Lo Bianco (1908)

\* Pelseener evidently derived his information from Kollmann (1876), who did not seem to be very clear whether his animals were fighting or mating, and ignored the more recent and accurate account of Racovitza (1894a).

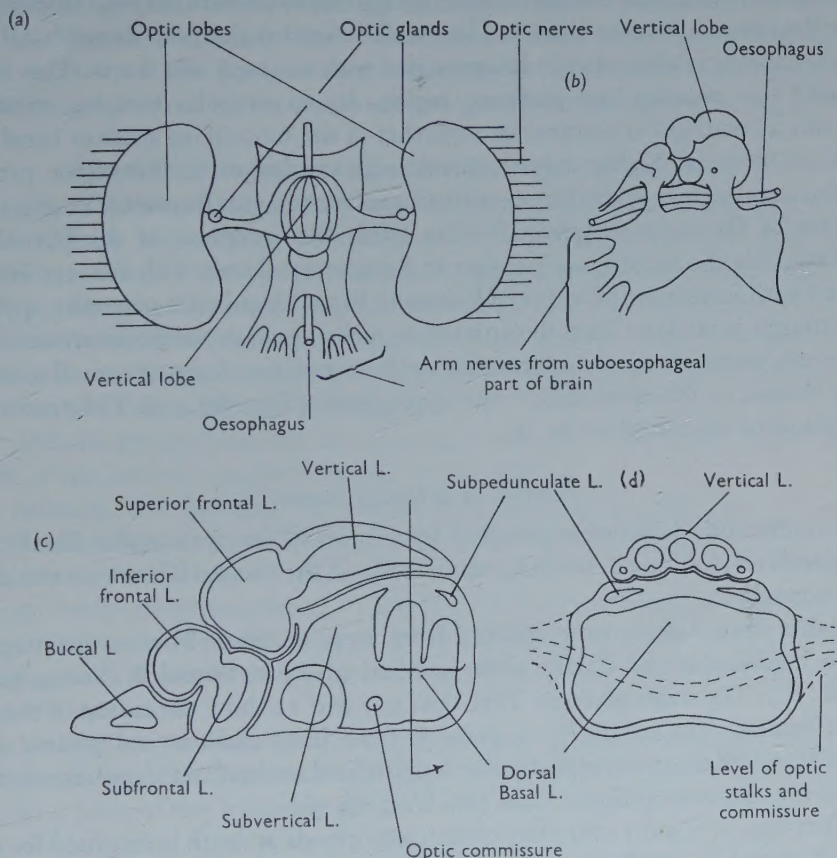
records an octopus of 100 g. from the Naples aquarium that grew to 410 g. in 6 weeks and our own animals of approximately 500 g. not uncommonly doubled their weight during 3 months of experiments in which they were fed four 10 cm. sardines daily, by no means their full capacity. It seems probable that in the sea the growth of octopuses is, in the normal course of events, limited mainly by the availability of food. Octopuses are solitary animals and one would expect to find a considerable scatter in the size of individuals from broods hatched at the same time. The only clue to the age of our experimental material is therefore the availability throughout the year of animals of the 400–500 g. size wanted for training experiments. In three successive years in Naples animals of this size were particularly difficult to obtain during the latter part of January and February, while individuals of up to 200 gr. or of over 1000 g. were relatively common; in October–December it was often hard to get animals of less than 700 g. These impressions are based upon deliveries to the laboratory and periodic visits to the local fishmarkets and cannot be quantified since we were deliberately selecting animals from a limited size range and kept no record of animals that we did *not* accept for use in experiments. The observations do nevertheless suggest that animals of 500 g. used in midsummer were in all probability spawned during the previous summer and if left in the sea would have attained a weight of 1000 g. in the following year.

In any batch of animals brought into the laboratory the females tended to be rather smaller than the males and to grow more slowly in the aquarium under exactly the same conditions. A similar state of affairs has been reported for *Loligo vulgaris* from the North Sea (Tinbergen & Verwey, 1945) where the females grow more slowly and reach sexual maturity later than the males; and among *Sepia* collected and exhibited in the Naples aquarium males were consistently larger than females (they were also more numerous, but this is because females are retained by the fishermen to catch more males (see Boycott, 1958)).

Out of 465 animals used in experiments on tactile discrimination, 251 were males and 214 females. We deliberately selected females during one short period of this work, but it is unlikely that this made as much as 5% difference to the totals. Assuming that there was no selection of males and females by the fishermen (unlikely, since the fisherman who supplied most of the material was unable to distinguish the sexes with any reliability, although he claimed to be able to do so), these figures indicate a sex ratio approaching 1:1, with, if anything, a preponderance of males. Crew (1927) quotes a sex ratio of 1 male to 3 females for *Octopus* at hatching as 'having met with general approval'. Presumably these figures do not refer to *O. vulgaris*; in order to get such information it would be necessary to rear the animals until their sex organs differentiated, as Montalenti & Vitagliano (1946) have done with *Sepia* (where, incidentally, they found a sex ratio of 1:1). Larval *O. vulgaris*, unlike *Sepia* which hatches from a much larger egg, are minute and pelagic and so far as we are aware nobody has yet succeeded in keeping them for more than a very few days. It seems therefore, improbable that Crew's figure refers to *O. vulgaris* and there is no need to suppose the very heavy selective mortality of females during the first year of growth that it would imply if correct.

*Location and nomenclature of parts of the brain discussed*

The brain of *Octopus* consists of a central mass surrounding the oesophagus broadly divisible into sub- and supraoesophageal regions, each of which is in turn divisible into a number of clearly defined lobes. The whole structure is enclosed in



Text-fig. 3. Location of parts of the brain mentioned in the text. (a) shows the brain as it would be seen from above after removal of the cartilaginous brain case, and (b) is a longitudinal vertical section through this. (c) shows further detail of the supraoesophageal part of the central mass and may be compared with Pl. 1a, which is a photograph of the same thing. (d) is a transverse section through the supraoesophageal lobes at the level indicated on (c) and corresponds to Pl. 1b. (c) and (d) are used as a basis for the standard diagrams used to record the extent of lesions in Text-figs. 5, 7 and 11. The dimensions of these parts naturally depend upon the size of the animal concerned. In an octopus of 500 g., section (c) would be approximately 4 mm. long.

a cartilaginous box except for the optic lobes which project from either side of the supraoesophageal mass. Text-fig. 3 shows the location and relative sizes of these parts. Within the supraoesophageal mass a number of lobes may readily be distinguished. For present purposes we are concerned directly only with the vertical lobe and the structures immediately underlying it, the subpedunculate and

dorsal basal lobes.\* The former is a discrete bilateral structure having its own walls of cells and neuropil (Pl. 1a, b) connected by tracts with the neuropil of the dorsal basal lobe. It is also the source of nerves running to the subpedunculate tissue in the orbits (Boycott & Young, 1956) and possibly of nerves to the optic glands (see p. 23); it is therefore of particular interest as being the only part of the supraoesophageal mass, other than the buccal lobe, giving rise to efferent nerves. Immediately below the subpedunculate lobes is a less well defined region, the dorsal basal lobe, characterized by islands of cells intermingled with neuropil and tracts. This lobe is separated into anterior and posterior regions by an irregular hanging curtain of cells, and its neuropil is continuous with that of the underlying median basal lobe. The basal lobes are higher motor control areas and lesions in this region produce deficiencies in posture and in the co-ordination of movement (Boycott & Young, 1950). The rest of the supraoesophageal lobes (with the exception of the buccal lobe which controls the mouthparts) appear to be concerned only with sensory integration and with learning; the effect of lesions to these lobes is not generally apparent until attempt is made to train the animals to make visual or tactile discriminations.

The only non-nervous structures that need be mentioned are two small spherical bodies located on the optic stalks—the optic glands (Text-fig. 3a). The anatomy of these glands is discussed on p. 18.

#### *Method of mapping lesions*

Where the optic lobes were removed by section of the optic stalks distal to the optic glands no checking other than examination of the excised lobes was considered to be necessary.

All other brain lesions were checked from serial sections. These were prepared using a modification of Cajal's silver method given in Sereni & Young (1932, method 'B'). The maps given in Text-figs. 5, 7 and 11 show the extent of the part removed assessed as accurately as possible from these sections and plotted upon a pair of standard diagrams representing longitudinal median and transverse sections through the supraoesophageal lobes (see Text-fig. 3).

In Text-figs. 5, 7 and 11 the state of the optic glands at death is recorded for each operated animal alongside the transverse map of the lesion made. R and L indicate enlarged and r, l normal sized optic glands on the right and left sides respectively. Where the condition of the gland was not recorded this is indicated —; absence of a record generally indicates that the optic gland was normal in size.

#### EXPERIMENTAL RESULTS

It will be shown below that section of the optic nerves or removal of certain parts from the supraoesophageal lobes from the brain of *Octopus* causes enlargement of

\* The anterior and posterior divisions of the dorsal basal lobe are equivalent to subvertical lobes 2 and 3 respectively in the nomenclature used by Boycott & Young up to and including 1956. The names have been changed (Boycott & Young, 1959), 'subvertical' being reserved for the old 'subvertical 1' to which go all efferent fibres from the vertical lobe (see Pl. 1a). The dorsal basal lobe has no direct connexion with the vertical.

the gonad. This enlargement is essentially a ripening process and the increase in size of the ovary in female animals is proportionally much greater than that of the testis in males, the ovary becoming relatively enormous as it ripens prior to egg-laying. Most of the experimental work has therefore been done with females, where enlargement of the ovary produces a quicker and more sensitive means of detecting changes arising from the experimental treatments than enlargement of the testis in males. Because of this, experiments with females will be dealt with first, followed by an account of the fewer similar experiments with males showing that the mechanism of control of maturation of the gonad is essentially the same in both sexes.

*Summary of experimentally induced enlargements of the ovary*

Text-fig. 1*b* shows the size of the ovary in seventy-two control animals with body weights of from 200–1000 g. In no case was the gonad/body weight ratio greater than 0.0032. When a survey was made of 105 animals kept for more than 7 days after lesions had been made to the supraoesophageal lobes of the brain and/or after the optic nerves had been cut it was found that in sixty-nine of them the gonad/body weight ratio was larger, sometimes very much larger, than this maximum. The experimental animals fall into 3 categories:

- (1) Animals having brain lesions including removal of the vertical lobe and of certain of the tissues lying immediately underneath it.
- (2) Animals with the optic nerves cut or the optic lobes removed.
- (3) Animals having central lesions of various types as well as having the optic nerves cut or the optic lobes removed.

These results are summarized in Text-figs 4 and 6 from which it can be seen that where enlargement is produced at all it is commonly very considerable, the ovary increasing from about 1/500th to as much as 1/5th of the total body weight within 6 weeks of operation.

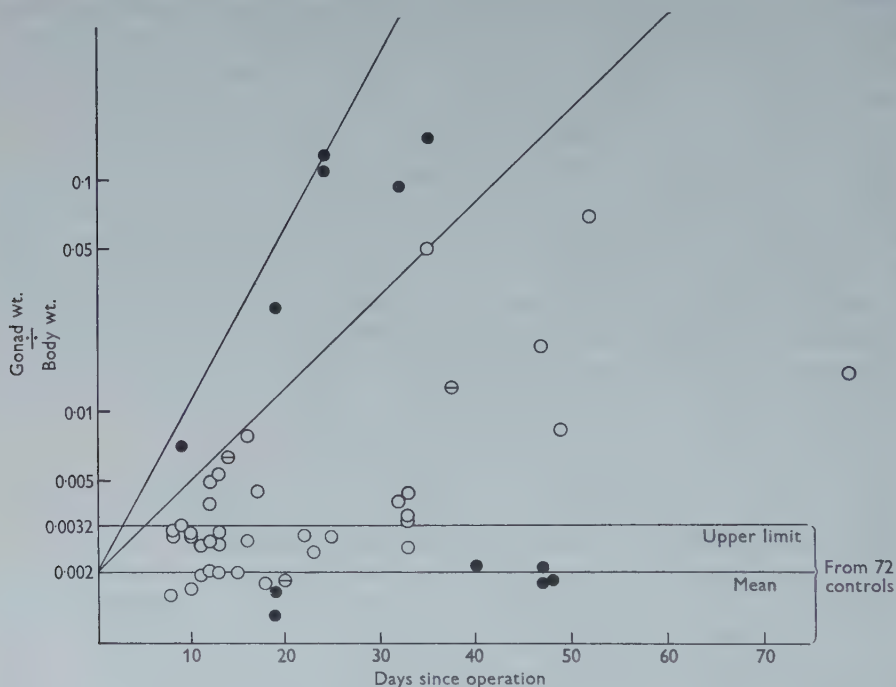
It is convenient to deal with the results in these categories separately although, as will be shown later, the results in category 3 can be reclassified into categories 1 and 2 on a basis of the lesions made.

*Category 1. Animals with central lesions and intact optic nerves*

Twelve animals taken from a long series of experiments by Boycott & Young in which part or the whole of the vertical lobe was removed in connexion with visual training experiments. Octopuses treated in this way were generally found to have ovaries of quite normal size when killed 2–6 weeks after operation but in a small proportion of cases very large ovaries were found, and the present sample of twelve animals includes six such cases as well as six of the more typical animals in which the ovary was not enlarged (Text-fig. 4). Maps of the lesions found in this sample of twelve animals are given in Text-fig. 5. In every one of the octopuses with enlarged ovaries the lesion was found to extend beyond the limits of the vertical lobe and to include removal of the subpedunculate lobe plus a variable amount of damage to the roof of the dorsal basal lobe on at least one side, a type of lesion not found in any of the animals with normal sized ovaries.

*Category 2. Blinded animals without central lesions*

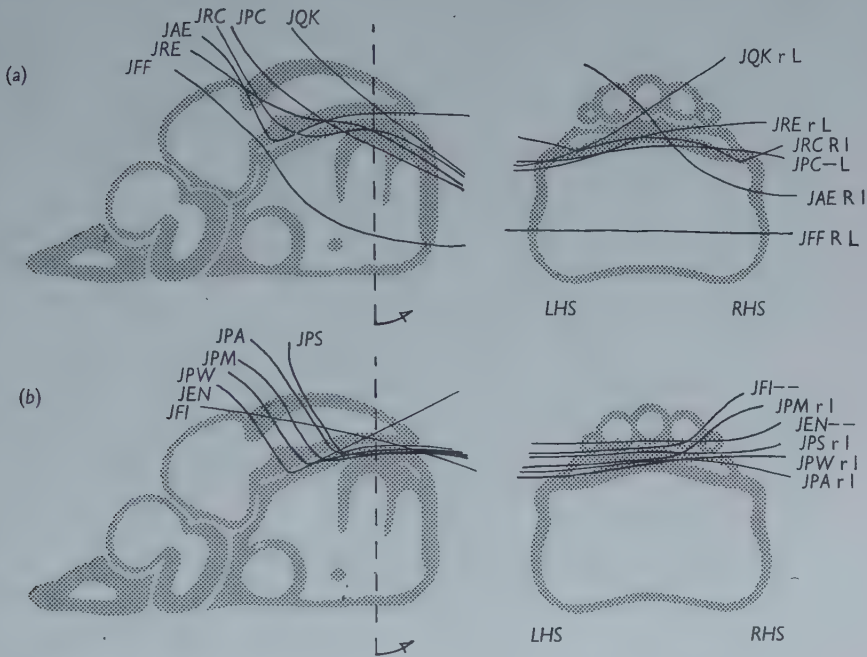
Octopuses were blinded by bilateral section of the optic nerves (thirty-five animals) or by removal of the optic lobes after section of the optic tracts distal to the optic glands (three animals, making thirty-eight in all). Sixteen of these animals (fourteen optic nerve sections and two optic lobe removals) had ovaries greater



Text-fig. 4. Enlargement of the ovary following brain lesions (●) or optic nerve section (○), being the results from experimental categories 1 and 2 (the latter including three animals from which the optic lobes were removed, indicated (⊖)). The gonad to body weight ratio is plotted on a log scale ranging from 1/10 to 1/1000th of the body weight, which includes that of the ovary. Maps of the brain lesions made in the twelve category 1 animals are given in Text-fig. 5 *a* and *b*.

than the maximum size found in controls (Text-fig. 4). Of the remaining twenty-two, most of which were kept for comparatively short periods after operation, only five had ovaries smaller than the mean control size, and it seems reasonable to suppose that the results with these five were due to incomplete operations. The maximum rate of enlargement of the ovary found in blinded animals is considerably lower than the maximum found in category 1, and there is a rather greater scatter in the individual rates of enlargement. At its most rapid, enlargement of the ovary following optic nerve section led to a 20-fold increase in the weight of the ovary within 5 weeks of operation; at the other end of the scale animals operated for just as long had ovaries only slightly larger than the maximum size found in controls. It is not clear why there should be this considerable variation and although reasons are given below for believing that the effect of blinding on any individual animal is

'all-or-nothing' in so far as ovarian enlargement is concerned, it remains possible that the scatter of rates is a reflexion of the degree of completeness of the operations. It is unfortunately almost impossible to check the completeness of optic nerve section; tests in which no visual reactions could be elicited proved on post mortem dissection to be unreliable, and dissection itself is an uncertain check because of the ease with which bundles of optic nerves remaining are overlooked or destroyed in

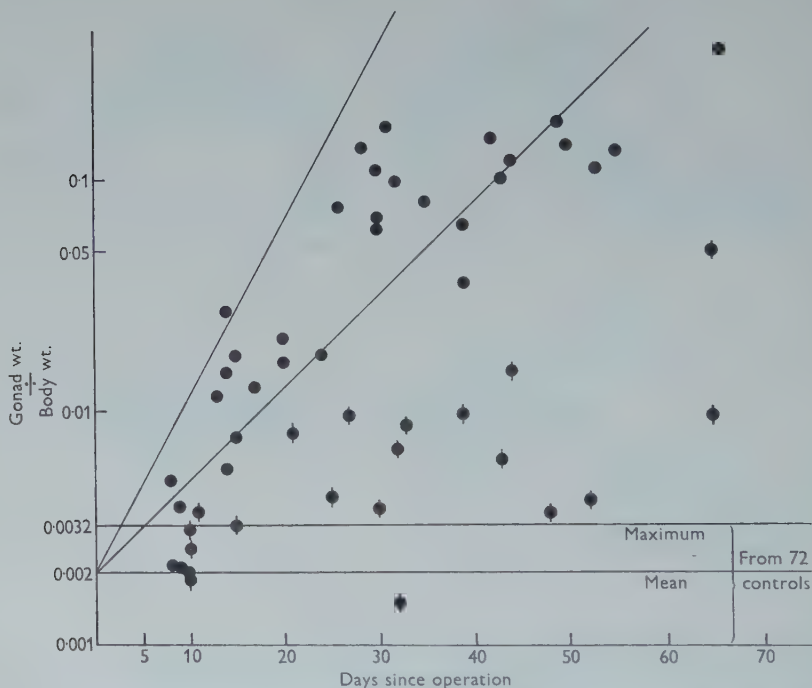


Text-fig. 5. Maps of lesions in category 1 experiments, prepared from serial sections, (see p. 8). (a) Lesions in six animals that had greatly enlarged ovaries; (b) in six animals with normal-sized ovaries. In all six of the octopuses with enlarged ovaries the lesion encroaches upon the dorsal basal lobe and includes the subpedunculate lobe on at least one side. These areas were not destroyed in any of the six animals where the ovary remained normal in size. In each case the condition of the right and left optic glands is indicated as follows: R and L—enlarged glands, r and l—normal glands; — indicates that the condition of the gland was not recorded (in most cases this means that it was normal in size).

searching amongst the flocculent 'white body' (Cazal & Bogoraze, 1943) that fills the orbit. This means that we cannot be absolutely certain that all the optic nerves were cut in any individual case and have no direct means of separating those animals in which the operations were complete from those in which the operations were very nearly complete. There is, however, an indirect method based on changes to the optic glands, which will be discussed later (p. 24); it is shown that blinding need only be complete on one side to be effective in causing gonad enlargement and this suggests that in the few cases where there was no increase in the size of the ovaries the optic nerves remained intact on both sides.

*Category 3. Blinded animals with central lesions*

This category includes all the rest of the experimental animals. Forty-seven out of a total of fifty-five had enlarged ovaries (Text-fig. 6). In twenty-one of these the ovary had enlarged at a rate comparable with that found in category 1 octopuses. This is faster than the maximum found in category 2 (blinded) animals and it seems fair to assume *a priori* that in these twenty-one cases enlargement was due to a central lesion and not to blinding alone. One would expect to find that these



Text-fig. 6. Enlargement of the ovary in category 3 experiments with animals that had been blinded as well as having central brain lesions. The results marked ● were from animals having lesions (Text-figs. 7a, b) including the area found to be critical in category 1 experiments with unblinded animals. The rest, marked ◆ were from animals having lesions of other types (see text and Text-fig. 7d). The results of three experiments in which animals were kept until they laid eggs are not included in this summary (but see Table 2).

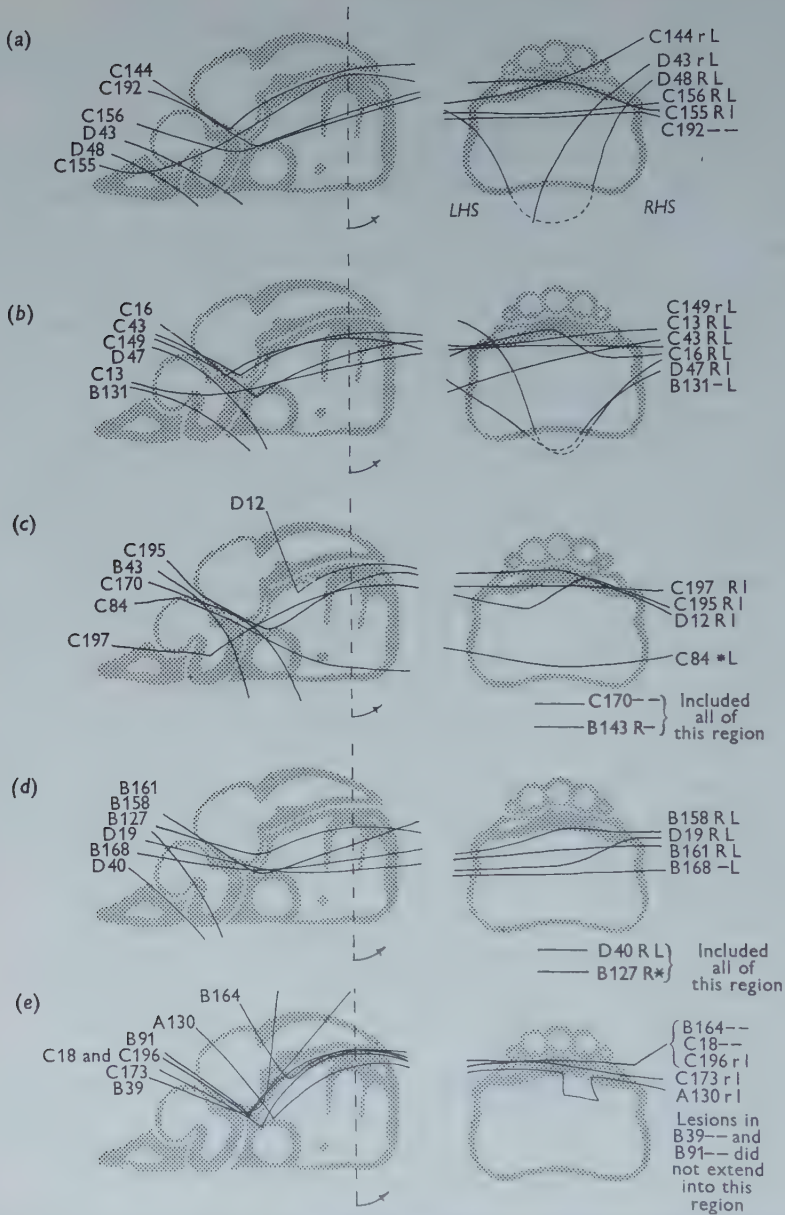
animals had brain lesions affecting the subpedunculate/dorsal basal area (found to be critical in category 1 experiments), and Text-figs. 7a, b and 11 show that this was indeed the case. There is one exception, octopus HBI, which has both optic tracts cut centrally; this operation is quite different from that made to remove the optic lobes in category 2 experiments since it cuts off the optic glands (Text-fig. 3a) from their nerve supply in the central part of the brain, a supply that is left intact by section peripheral to the optic glands. It will be shown below that this nerve supply originates in the area already found to be critically related to ovarian development

in category 1 animals, so that section of the optic tracts as carried out in the case of HBI may properly be compared with lesions of the sort shown in Text-fig. 5*a* in so far as any effect upon the gonads is concerned.

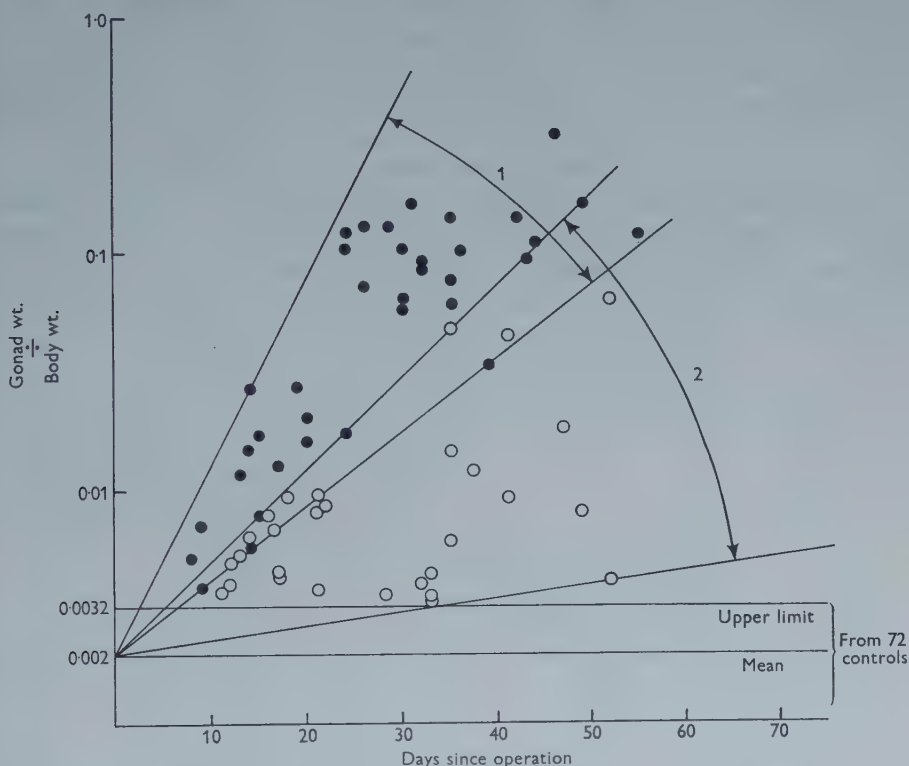
Since the most rapid rate of ovary development found in the forty-seven category 3 animals was not significantly greater than the maximum found in the six category 1 octopuses we can infer that the effect of blinding does not add to the effect of central lesions on gonad size.

The remaining thirty-four animals in category 3 had ovaries that were no larger than might be expected from blinding alone. In order to assess these results we must take into account the operational routine to which each individual was subjected and the nature of the lesions made to its brain. We already have a good idea of which central lesions cause gonad enlargement from the results in category 1 and from the animals showing particularly rapid enlargement in category 3, and we can subdivide these thirty-four animals into two groups on a basis of whether or not their lesions were of a type associated with gonad enlargement elsewhere. When this is done we find that out of the twenty-six animals with ovaries greater than the control maximum, twelve had lesions of the type associated with enlargement of the ovary elsewhere (Text-fig. 7*c, d*). The rates of enlargement found in this group of twelve animals were all towards the upper limit of the range found in category 2 (Text-fig. 6) and four of them were subjected to two successive operations, being blinded by optic nerve section up to 3 weeks before central lesions were made. In these four the final rate of gonad enlargement is a compound of two rates, and includes a considerable period of relatively slow increase after the first operation followed by a much more rapid increase after the second. When this is taken into account it is clear that the final gonad weight indicates a rate of enlargement following the second, central operation, fully comparable with that found in category 1 animals. The rates of increase in the remaining eight animals from this group fell well within the upper third of the range found in blind animals, and although in these cases the cause of enlargement must, strictly speaking, remain uncertain, the nature of the lesions made and the rates of enlargement found together indicate ovarian increase brought about by destruction in the usual subpedunculate/dorsal basal region.

This leaves fourteen animals out of the total of forty-seven with enlarged ovaries that had central lesions of types not found to be associated with enlargement of the ovary elsewhere. Nine of these animals had central lesions (not plotted) affecting the frontal inferior and subfrontal lobes only, one had the frontal superior removed, and the remaining four lesions confined to the vertical and superior frontal lobes (Text-fig. 7*e*). The rates of ovarian enlargement found in these animals all fell well within the range found in category 2 experiments as a result of optic nerve section alone, and it seems reasonable to suppose that these animals had big ovaries because they had been blinded rather than because of their central lesions. In so far as any effects of operation on ovarian development are concerned the animals in this group may properly be regarded as category 2 experiments, and their 'time since operation' measured from the date on which they were blinded.



In Text-fig. 8 the category 3 experiments are reclassified, according to whether ovarian enlargement was induced (or probably induced) by central brain lesions or by blinding alone and these reclassified results are plotted together with those already given in Text-fig. 4 (results from categories 1 and 2) to define more accurately the relationship between the rates of enlargement produced by the two alternative treatments.

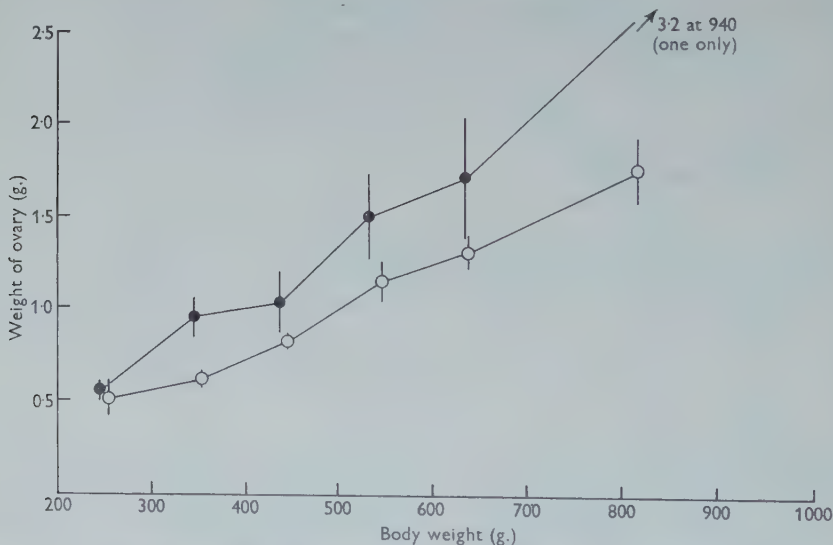


Text-fig. 8. A comparison of the rates of enlargement of the ovary found: (1) after brain lesions including the subpedunculate and part of the dorsal basal lobe (indicated ●); and (2) after optic nerve section (○). This summary includes the results from experiments in categories 1 and 2 already given in Text-fig. 4 as well as those from category 3 (animals which had the optic nerves cut and central lesions) reclassified on a basis of the lesions made (see text). Results from animals that laid eggs (see Table 2) are not included.

Eight octopuses out of the fifty-five in category 3 were kept for more than 7 days after operation without developing ovaries larger than the maximum size found in controls. Only two of these eight were kept for more than 10 days. One of these animals (C196NVB) had an ovary/body weight ratio of 0.0032—equal to the maximum size found in controls. The other (C173NVB) was kept for thirty-two days after optic nerve section; since its gonad/body weight ratio was only 0.0015 at the end of this period it must be supposed that in this case blinding was incomplete. Maps of the lesions in these animals are included in Text-fig. 7e—in neither case did the parts removed include the subpedunculate or dorsal basal lobes.

*Experiments with animals that were kept from 4 to 7 days after operation*

The weight of the ovary in animals that are killed within 3 days of operation does not differ significantly from that in unoperated animals. This ceases to be true after about 4 days, by which time the gonad has already begun to show signs of enlargement as a result of the treatments described above. In Text-fig. 9 the average weight of the ovary in seventy-two controls is compared with that in fifty-four animals killed 4-7 days after operation. Comparatively few of the latter were used in training experiments and the brains of most of them have not been sectioned to check the lesions made. No attempt therefore has been made to break down this data into categories and it is likely that at least some individuals had operations that would not have produced enlargement of the ovary. Nevertheless there is a significant difference between the mean weights in this group and in controls, indicating that enlargement of the ovary begins almost immediately after operation. At this stage there is no difference in the ovaries or in their ducts visible on dissection, although the optic glands (see p. 21) may already be noticeably enlarged.



Text-fig. 9. Enlargement of the ovary 4-7 days after operation. ○=summary of weights from seventy-two controls (see Text-fig. 1b). ●=summary of weights from fifty-four animals killed 4-7 days after operations in which they had been blinded and/or had central lesions made. Mean and standard error is given in each case for each 100 g. size group as in Text-fig. 1.

*Changes in the ovary and its ducts following operation*

Fifteen days after suitable central brain lesions have been made, and about twice as long after optic nerve section, the ovaries of experimental animals are noticeably enlarged on postmortem dissection. Enlargement continues until the bodies of operated animals become visibly distorted by the ovary within (Text-fig. 2b). Five weeks after an operation involving removal of the subpendunculate lobe on at

least one side the ovary may constitute as much as  $1/5$ th of the total body weight. On dissection of such animals it is found that the swollen ovary occupies the whole of the hind part of the body (Text-fig. 2*d*) considerably displacing the other organs which appear by contrast unusually small (weights taken from about thirty animals show, however, that the kidneys, ctenidia and hearts at least remain quite normal in size). The ovary itself, now pale yellow in colour, is crammed with small ( $1 \times 3$  mm.) eggs, all of the same size which is approximately that of the eggs when laid. As the ovary increases in size so do its ducts. These become swollen to several times their normal diameter, and the oviducal glands increase greatly in circumference, becoming almost disk-like, ribbed and brown in colour at the centre (Text-fig. 2*d*).

*Egg-laying and care of eggs by operated animals*

Three octopuses were kept until they laid eggs after operation. Particulars of these three animals are given in Table 2, and details of the brain lesions concerned included in Text-fig. 11. In two out of these three cases (C27 and C28) the eggs were examined at intervals and those laid by C28 were found to be fertile, developing until the eyes of the larvae were clearly visible, at which stage a failure in the circulation system unfortunately killed both eggs and parent. We have no record of the sex of the octopuses kept in the tanks adjoining those of C27 and C28, so we do not know when the eggs from C28 were fertilized or whether the infertility of C27 was a result of isolation from male octopuses.

Table 2. *Particulars of three octopuses that laid eggs after brain operations.*  
*The lesions concerned are mapped in Text-fig. 11*

(The weight of the eggs laid by C27 and C28 was not recorded.)

Reference no.	Interval between operation and egg-laying	Weight of eggs laid (g.)	Weight of ovary at death (g.)	Weight of animal at death (g.)
C9	39 days since optic nerves cut and central lesion made	227	8.0	700
C27	64 days since optic nerves cut, 20 days since central lesion made	—	117.7	890
C28	69 days since optic nerves cut, 25 days since central lesion made	—	107.0	800

The behaviour of these three animals was very similar. About a week before egg-laying they began to feed irregularly, sometimes rejecting food (sardines and crabs) that they had hitherto accepted eagerly. As the time of oviposition approached the animals showed a tendency to remain in one particular well-aerated place in their tanks, in the angle between the side of the tank and the water surface. Breathing was deeper and more rapid than usual. Eggs were laid, in bunches attached to the side of the tank, at intervals over a period of about a week; during this period and subsequently the animal did not leave the eggs except very occasionally and then only for a few seconds at a time (generally to reject food presented

to it). Although in the normal way octopuses will at first attempt to eat any unfamiliar object presented to them (Wells & Wells, 1956), objects presented to brooding animals were invariably rejected without first being passed to the mouth. In many cases objects such as crabs and pieces of sardine were carried to the opposite extremity of the tank before being dumped and blown away with vigorous jets from the funnel. Brooding animals were never observed to feed, but evidently did so occasionally since sardines left in their tanks were sometimes eaten overnight. These observations agree closely with accounts of egg-laying and brooding by normal *O. vulgaris* (Lo Bianco, 1908; Monticelli, 1921; Heldt, 1948; Vevers, 1959) and with Batham's (1957) detailed account of *O. maorum* which appears to behave like *O. vulgaris*. Taken together with the fact that these operated animals can evidently lay viable eggs, these behavioural similarities strongly support the view that the experimental treatments discussed in this account cause the gonad to mature in a manner essentially normal although precocious.

#### *Enlargement of the optic glands*

The optic glands are small (1 mm.) pale yellow bodies lying on the optic stalks (Text-fig. 3a). These bodies have in the past been variously identified as glands or as nervous tissue, the spherical glands which are readily visible on dissection being confused with the olfactory and peduncle ganglia seen in sections (see review by Boycott & Young, 1956). Enlargement of the ovary following optic nerve section or central brain lesions is always accompanied by changes to the optic glands, which become bright orange in colour and may increase to more than ten times their normal size.

#### *Histological methods and histology of the optic glands in control animals*

Most of the optic glands studied were fixed in neutral formalin in seawater and all measurements of glandular and nuclear volumes quoted are taken from such material embedded in paraffin and sectioned at  $8\mu$ . A small number of normal glands were fixed in Susa, Bouin and Flemming; stains used were Heidenhein's iron haematoxylin, Azan, the Feulgen stain for DNA and Unna's methyl green-pyronin, the last two being used only on formalin-fixed material. A modification of Cajal's silver method (modification B) given in Sereni & Young (1932) was used to show the nerve supply.

The cells of the optic gland are arranged in solid masses and do not form vesicles; there are two types, chief cells and smaller supporting cells (Text-fig. 10a) (Boycott & Young, 1956). The chief cells have large nuclei, prominent nucleoli in finely granular nucleoplasm, and cytoplasm of irregular shape. With Azan, nucleoli and nuclear granules stain dark blue, the cytoplasm a paler cloudy mauve. The supporting cells have smaller, more regularly oval nuclei without prominent nucleoli; the nuclear granules which are larger than those of the chief cells stain bright red with Azan, while the rather scanty fibrous-looking cytoplasm stains a clear pale blue. The nuclei of the supporting cells, particularly the nuclear granules, stain intensely with Schiff's reagent following 15 min. N/HCl digestion at  $60^{\circ}\text{C}$ ., the colour being a bright reddish purple, in contrast to undigested controls which remain unstained.

In such preparations the nucleoli of the chief cell nuclei stain very faint pink, while the rest of the nucleus remains colourless. With methyl green-pyronin the nuclei of the supporting cells stain intensely, while the cytoplasm remains relatively pale, reactions that are not affected by ribonuclease digestion. Both nuclei and cytoplasm of the chief cells stain intensely with this stain and, in contrast to the results with the supporting cells, the uptake to stain is almost entirely eliminated by digestion with ribonuclease for 3 hr. at 60° C., only the nucleoli staining faintly thereafter.

The results with Schiff's reagent and methyl green-pyronin together indicate a relatively low concentration of DNA in the nuclei of the chief cells (where it appears to be limited to the nucleoli) and a much higher concentration of RNA in both nucleus and cytoplasm than in the supporting cells. It is likely, since high RNA content is characteristically associated with protein synthesis (see Brachet, 1957), that the chief cells secrete protein and that the supporting cells do not.

Measurements were made from the glands of six control animals weighing between 390 and 1140 g. and it was found that the ratio of glandular volume to body weight remains approximately constant over this range. It was also found that the mean volume of the chief cell nuclei rises with increase in body size so that the ratio of individual nuclear to glandular volume remains more or less constant (Table 3); this implies that the actual number of chief cell nuclei does not increase as the animal grows, a finding that is consistent with our failure to discover mitotic figures among the chief cell nuclei in any of the glands examined.

The nerve supply of the optic glands, which can be shown by degeneration experiments to originate in the subpedunculate/dorsal basal lobe area (p. 24), enters the gland by way of the olfactory lobe and immediately breaks up into a large number of fibres ramifying in the gland mainly in the fibrous tissue. An abundant blood supply to the gland can be demonstrated by injecting methylene blue into the dorsal aorta; the optic glands become more intensely blue than any of the surrounding tissues (Boycott & Young, 1956).

#### *Histology of enlarged glands*

When the optic glands enlarge there appear to be no changes in the size, number or staining properties of the supporting cells and it is concluded that these play no part in secretion, their function being limited to the provision of a connective tissue framework, as in any case seems likely from their staining properties in the normal gland.

The individual chief cells, on the other hand, increase very considerably in volume as the gland enlarges (see below); their nuclei, nucleoli and nuclear granules all enlarge while the cytoplasm increases in volume and becomes vacuolated. These changes are progressive and occur both after blinding by optic nerve section and after central lesions of the type already described. Changes after blinding are relatively slow (Table 3) but appear to be qualitatively identical with those brought about by central lesions.

Table 3. *Measurements from optic glands at various periods after operation*

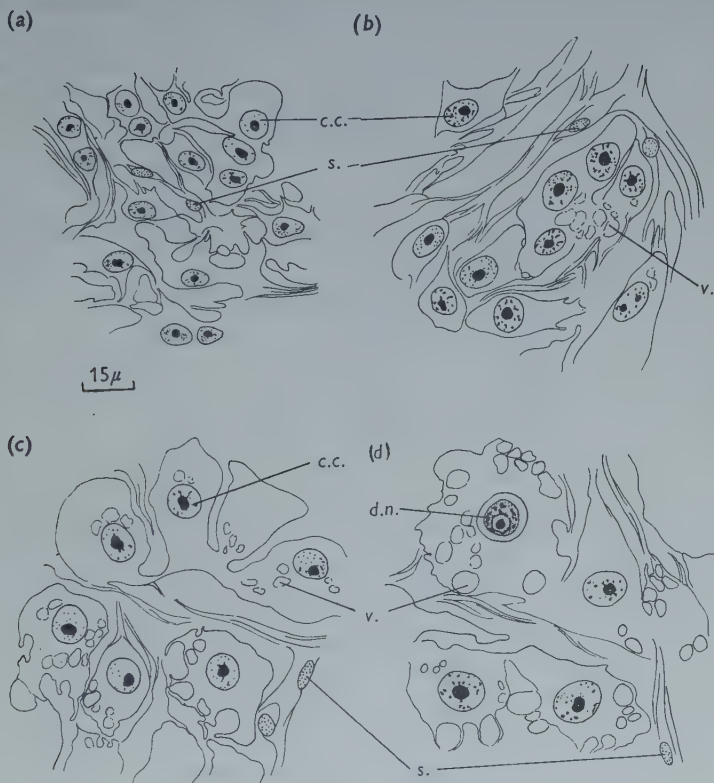
(Animals C 197, C 52, C 144 and C 51 had unilateral central lesions (those of C 197 and C 144 are plotted in Text-fig. 7) as well as being blinded by section of the optic nerves, and occur twice in this table, the optic gland on one side serving to show the effect of blinding alone while that on the other optic stalk demonstrates the more rapid consequences of a suitable central lesion. The volume of the whole gland (B), which is approximately spherical, is calculated from a mean of two diameters at right angles, that of the chief cell nuclei (C) from a mean of 20 or 40 measurements from individual cells.)

Reference no.	Sex	Days since operation	Body wt. (A) (g.)	Volume of optic gland (B) (mm. <sup>3</sup> )	Volume of chief cell nuclei (C)( $\mu^3$ )	B/A $\times 1000$	B/C $\times 100$
Controls							
C 137	+O <sub>3</sub>	—	390	0.25	230	0.64	0.11
C 175		—	420	0.18	180	0.43	0.10
C 47		—	560	0.25	210	0.45	0.12
C 178		—	630	0.44	260	0.70	0.17
B 138		—	710	0.29	330	0.41	0.09
B 141		—	1140	0.44	270	0.39	0.16
Mean						0.51	0.13
Blind animals							
C 197	O <sub>3</sub>	15	460	0.21	290	0.46	0.07
C 52		21	740	0.46	360	0.62	0.13
C 144		31	490	0.27	190	0.55	0.14
C 51		37	1070	0.46	290	0.43	0.17
D 16		52	570	0.93	330	1.64	0.28
C 5		100	630	1.04	360	1.66	0.29
Animals with central lesions							
C 197	O <sub>3</sub>	15	460	0.70	360	1.52	0.20
C 52		21	740	3.05	660	4.15	0.46
C 144		31	490	2.30	490	4.70	0.47
C 51		37	1070	5.85	1100	5.50	0.54

In no instance were mitotic figures observed in enlarged glands and, as there was no accumulation of material in the spaces between the cells, increases in the size of the glands must be attributed to increase in the size of their individual chief cells. As Table 3 shows, this increase may be very considerable since the volume of an optic gland can rise to more than ten times its normal value within 5 weeks of operation. The bulk of this increase is evidently cytoplasmic since the rise in volume of the individual chief cell nuclei does not keep pace with the rise in volume of the gland as a whole (Table 3), an observation that is in keeping with histological evidence indicating accumulation of material in vacuoles in the cytoplasm of the enlarged chief cells (Text-fig. 10*b, c, d*). It would appear that under these conditions the gland cells secrete material faster than it can be carried away in the bloodstream, and that the disproportionate increase of cytoplasm is due in the main to accumulation of secretory product.

Where a considerable time has elapsed since operation (as in the case of C 51, see Table 3) occasional nuclei can be found in which the nucleolus has more or less broken down and the nuclear granules, further increased in number and size, have become arranged in a dense ring round the periphery of the nucleus. In extreme

cases the nucleus appears to be surrounded by a vacuole (Text-fig. 10*d*). Such nuclei are always relatively small and may represent a final stage in the cycle of changes beginning with enlargement of the nucleus, nucleoli and nuclear granules, and ending with collapse of the nucleus and disintegration of the nucleoli.



Text-fig. 10. Drawings from sections of optic glands fixed in neutral formalin and stained with Azan. (a) From a control animal (B 138 in Table 3); (b) from an octopus blinded by optic nerve section 100 days previously (C 5); (c) from an animal in which a central lesion including the subpedunculate lobe was made 21 days previously (C 52); and (d) from an animal with a similar lesion made 37 days before (C 51). *c.c.*, chief cells; *s.*, supporting cells; *v.*, vacuoles of secretory material; *d.n.*, disintegrating nucleus. All glands from male animals, and all drawn to the same scale.

#### *Relation between enlargement of the optic glands and enlargement of the gonad*

When the gonad is enlarged it is invariably found that one or both of the optic glands is enlarged as well. Lesions that cause the optic glands to enlarge invariably also cause enlargement of the gonad. This could be coincidental, although it is difficult to imagine why cuts severing the nerves innervating the glands should lead to enlargement of the sex organs unless the optic glands are in some way responsible. To establish the causal relation between secretion by the optic glands and maturation of the sex organs a further series of experiments was carried out in which the optic glands were removed before treatments known to cause gonad enlargement.

The optic glands were removed from five female octopuses in operations that included removal of the optic lobes by cuts peripheral to the site of the optic glands and removal of the greater part of the central supraoesophageal mass, leaving only parts of the frontal inferior, subfrontal and buccal lobes.\* In a further two females the optic lobes alone were removed. These were all treatments that in the normal way would certainly have produced a noticeable enlargement of the gonad within

Table 4. *Effect of removal of the optic glands on the size of the gonad*

Males

Reference no.	Gonad wt. (g.)	Body wt. (g.)	Gonad wt. above or below mean for controls	Days since operation
A. Unblinded animals without central brain lesions				
A 176	5.6	660	Below	33
A 192	6.8	920	Below	47
A 195	3.5	360	Above	8
A 197	2.8	590	Below	44
B. Animal with the optic lobes removed				
B 175	1.4	290	Below	30

Females

Reference no.	Gonad/body weight ratio	Ratio above or below mean for controls	Days since operation
A. Unblinded animals without central brain lesions			
A 183	0.0024	Above	17
A 193	0.0032	Above	47
C 62	0.0022	Above	45
C 67	0.0014	Below	45
B. Animals with the optic lobes removed			
C 44	0.0021	Above	48
C 56	0.0018	Below	50
C. Animals with large central lesions			
B 173	0.0022	Above	10
C 85	0.0027	Above	14
C 116	0.0014	Below	32
C 119	0.0011	Below	55
C 121	0.0014	Below	55

two weeks, yet in no case was the gonad weight at death outside the control range even though some of the animals had been kept for as long as 8 weeks after operation (see Table 4). Four out of the seven females actually had ovaries smaller than the mean size found in controls.

Removal of the optic glands does not lead to any changes in the behaviour or condition of the experimental animals that we have been able to observe, a finding

\* Maps of these lesions are not included in this account. All involved removal of the whole of the area shown in the standard T.S., being of the same general type as those in B 143 and C 170 (Text-fig. 7c).

that agrees with Sereni's (1930) and Callan's (unpublished, quoted Boycott & Young, 1956) observations, nor does it lead to a significant regression in the size of the gonad, at any rate in female octopuses. In Table 4 are listed the ovary/body weight ratios from 11 animals kept for varying periods after removal of the optic glands; the mean ovary/body weight ratio from these animals is almost exactly the same as in controls (0.0022 against 0.0020).

The situation is less clear in the case of males, where only five experiments were made. In four of these, kept for a period of from 4 to 7 weeks after operation, the testis was markedly smaller than the mean for controls of similar size; the remaining animal, with a testis larger than the mean control size, was kept for only 8 days after operation.

The findings outlined above would appear to be exactly what one might expect from animals in the size range (200–1000 g.) studied. Optic gland removal produces no regression of the gonad in females because the ovary is in any case undeveloped. In males where the gonad is normally ripe it seems that some decrease in size is caused, although because of the small number of experiments it is impossible to be certain of this. This implies that the optic glands secrete to some extent all the time (an implication that is borne out by the histological data (p. 19)) and that a lower level of secretion is required to ripen the testis than the ovary.

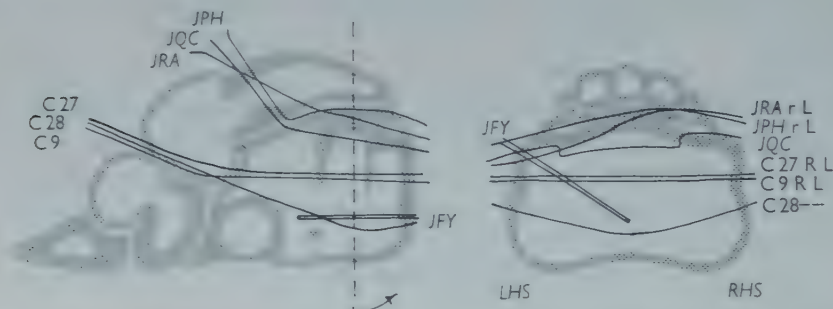
#### *Conclusions from optic gland enlargement following central lesions*

As has been pointed out above, rapid enlargement of the gonad over and above the rate found in blinded animals is always associated with lesions including the subpedunculate lobe and a variable amount of damage to the dorsal basal lobe on at least one side, a type of lesion that is also invariably accompanied by enlargement of the optic glands (or gland if the lesion is unilateral) (Text-figs. 7 and 11).

From the present series of experiments we cannot be sure whether it is removal of the subpedunculate or damage to the dorsal basal lobe that is responsible for enlargement of gland and gonad. The subpedunculate lobe is a well-defined tissue and it is possible to be reasonably certain when it is absent and when parts remain. Unfortunately its removal inevitably damages also the dorsal basal lobe and it is much more difficult to assess whether or not parts of this are damaged or missing. The dorsal basal lobe has a thick roof with an ill-defined upper margin that wraps round the forward-reaching lateral extensions of the subpedunculate lobe above, and breaks up into islands of cells mixed with tracts below (Pl. 1*b*). Since lesions are always followed by a certain amount of distortion of the remaining structures it is exceedingly difficult to map the area of tissue destroyed in this region at all accurately. This is particularly true in animals kept for a considerable period after operation, as were those used for the present series of experiments, and it means that although some damage to the dorsal basal lobe seems always to be found in animals having enlarged ovaries, the exact extent of this damage and whether it is itself the cause of ovarian enlargement must remain at present uncertain.

Lesions of the type associated with gonad enlargement cause degeneration of the nerves ramifying among the cells of the optic glands, and tracts, originating in the

subpedunculate dorsal basal area, can be traced along either side of the brain until they run out along the optic stalks to the optic glands. Section of the tract on either side is followed by the appearance of degeneration granules in the optic gland on that side (and only on that side) within 24–48 hr. (Pl. 2*b* and Text-fig. 11) and later by enlargement of the chief cells in the gland. Since removal of the optic gland is not followed by gonad enlargement the effect of severing this nerve supply cannot be attributed to the failure to release secretion by the glands. The nerve supply must therefore be inhibitory, limiting secretion in the intact animal.



Text-fig. 11. Lesions: (1) in animals that laid eggs—C9, C27 and C28, particulars of which are given in Table 2; (2) in animals used for nerve degeneration experiments, degeneration granules being found in the optic glands of *JQC* and *JFY* (LHS only) 48 and 24 hr. respectively after operation; a photograph of the condition in the LHS gland of *JQC* is included in Pl. ; (3) in males where the optic glands were enlarged unilaterally (in these two cases on the LHS only)—*JPH* and *JRA*. Condition of the optic glands is indicated as in Text-fig. 5.

#### *Conclusions from optic gland enlargement following blinding*

The optic glands also enlarge when the optic nerves are cut or the optic lobes removed by cuts peripheral to the optic glands. The histological changes to the glands, though rather slower, appear to be identical with those following removal of the tissues overlying the dorsal basal lobe (Text-fig. 10) although neither method of blinding interferes directly with the innervation of the optic glands. As with central lesions, the effect of blinding can be unilateral, the optic gland enlarging only on the side where the optic nerves are cut. In no instance did careful dissection reveal optic nerves remaining on a side where the optic gland enlarged, while several cases in which the optic gland remained normal in size and colour on one side were found to be attributable to incomplete section of the optic nerves on that side. Since unilateral section of the optic nerves leaves the unoperated side normal in size and colour the effect of blinding on the glands must be mediated through the nervous system and not by means of any intermediate hormonal mechanism.

It seems likely that this nervous control involves reduction of the inhibitory effect of the central subpedunculate dorsal basal area rather than excitation of the glands via innervation from another source. The optic glands are placed on the optic stalks so that innervation can come only from the optic lobes or the central

brain mass. Section of the optic stalks centrally to the optic gland causes very rapid enlargement of gland and ovary (animal HBI, p. 12) and therefore presumably does not cut off an excitatory innervation as well as the inhibitory supply from the subpedunculate/dorsal basal lobe area. Removal of the optic lobes by cuts peripheral to the optic glands produces enlargement of the gonad comparable to that produced by section of the optic nerves (p. 10) and excludes the possibility of excitatory innervation from the other direction.

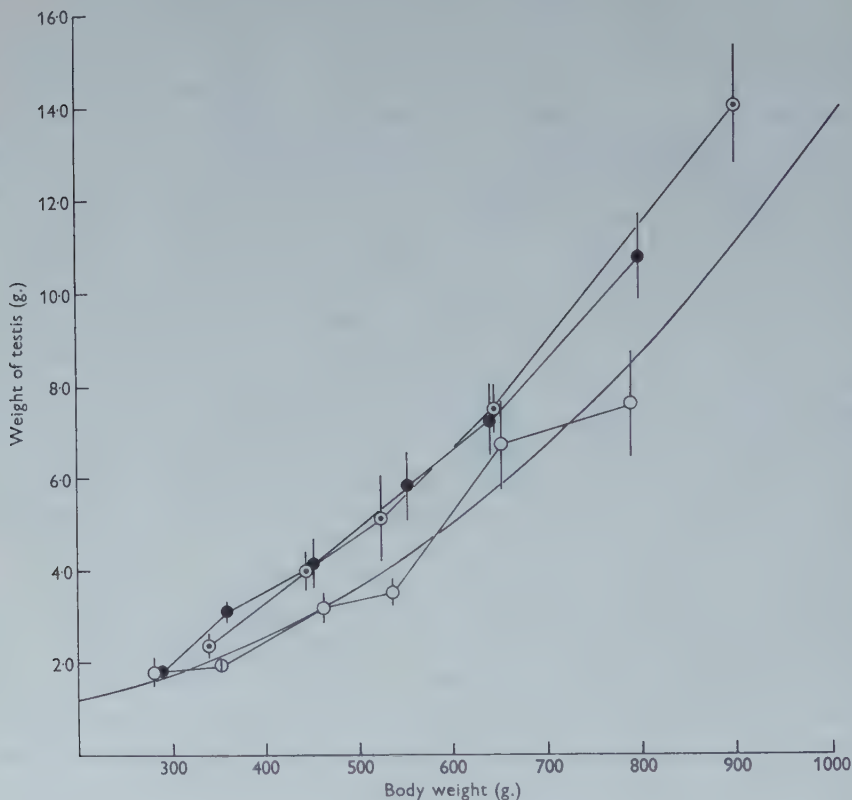
*Experimentally induced enlargement of the testis*

Section of the optic nerves or removal of the dorsal basal lobe from the brain of male octopuses causes the optic glands and testes to enlarge. The changes in the optic glands are as in females but the increase in size of the gonad is relatively much smaller, presumably because the testes are already ripe at the start of the experiment in all but the smallest of the animals used. The testis in any case never attains a size comparable with that of the ripe ovary and even in the largest animals with ripe spermatophores forms only about 1/100th of the total body weight (Table 5). Because gonad enlargement is not as spectacular as in females it is more difficult to recognize when it has occurred and this, together with the considerable range of testis size in controls (Text-fig. 1*a*) makes it impossible in most individual cases to be sure whether or not the testis is larger than usual. It is found, however, that the average size of the testis in males subjected to treatments known to cause enlargement of the ovary in females is significantly greater than that in controls. In Text-fig. 12 a comparison is made between the testis weight from 84 controls and 100 experimental animals blinded by optic nerve section or subjected to operations including removal of the central brain mass as well as optic nerve section. The two experimental groups respectively correspond to categories 2 and 3 from the experiments with females. The difference between experimentals and controls is clearly significant. There seems, on the other hand, to be no difference in the testis weights from animals in categories 2 and 3, and on this showing it could be argued that central lesions found to cause enlargement in females are without effect upon males. Enlarged optic glands occur, however, in unblinded males after central operations of the type associated with gonad and optic gland enlargement in females (see, for example, octopuses *JPH* and *JRA* in Text-fig. 11) and unless we suppose that the optic glands have a quite different function in males and females (which seems improbable) this must mean that central lesions can produce enlargement of the testis. The situation in the two sexes is therefore essentially similar, the state of the gonad being controlled by a central region in the brain acting on the optic glands.

*Results with animals larger than 1000 g.*

So far in this account only results from animals weighing between 200 and 1000 g. have been considered. This range was determined by the training experiments for which most of the animals were used, animals below 200 g. being inconveniently small for brain operations, and above 1000 g. being rather too large for the available tanks. Nevertheless a number of experimental animals, weighing

less than 1 kg. when brought into the laboratory, grew to weights of between 1 and 2 kg. in the course of the experiments. Most of these animals were males. Table 5 is a list of these animals, together with a few records of gonad weights from large octopuses that died in the Naples public aquarium, including one record from a female of 2 kg. (octopus 'Z') having an ovary weighing 45.0 g. There were visible, though not full sized, eggs in this ovary—the only record we have of a female in which the ovary appeared to be ripening without operational assistance. There are



Text-fig. 12. Enlargement of the testis as a result of brain lesions or optic nerve section. ○ = summary of weights from eighty-four controls (see Text-fig. 1 a). ◐ = summary of weights from forty-six blinded animals. ● = summary of weights from fifty-four animals with central lesions most of which had also been blinded. Mean and standard error is given in each case for each 100 g. size group as in Text-fig. 1.

thirty-five records in all, twenty-three from males and twelve from females, most of them animals with central lesions as well as having the optic nerves cut (category 3 experiments); a few had only the optic nerves cut or the optic lobes removed (category 2). These records have not been included in the main body of results because of the scarcity of controls above 1000 g.; they do, however, clearly agree in all important respects with those from the 200–1000 g. range, confirming that

Table 5. Results of experiments with animals larger than 1000 g.

(All weights in g. Gonad/body weight ratio is given for females in which the mean for controls below 1000 g. was 0.0020 and maximum 0.0032. Where more than one figure is given under 'Days since operation' there were two successive operations, at one of which a central lesion was made, and at the other the animal was blinded (indicated by B); the first figure is inclusive. Condition of the optic glands, where this was recorded, is shown as follows; R and L, right and left glands enlarged, r and l, glands not enlarged. \* Indicates a lesion including the subpedunculate/dorsal basal area on at least one side.

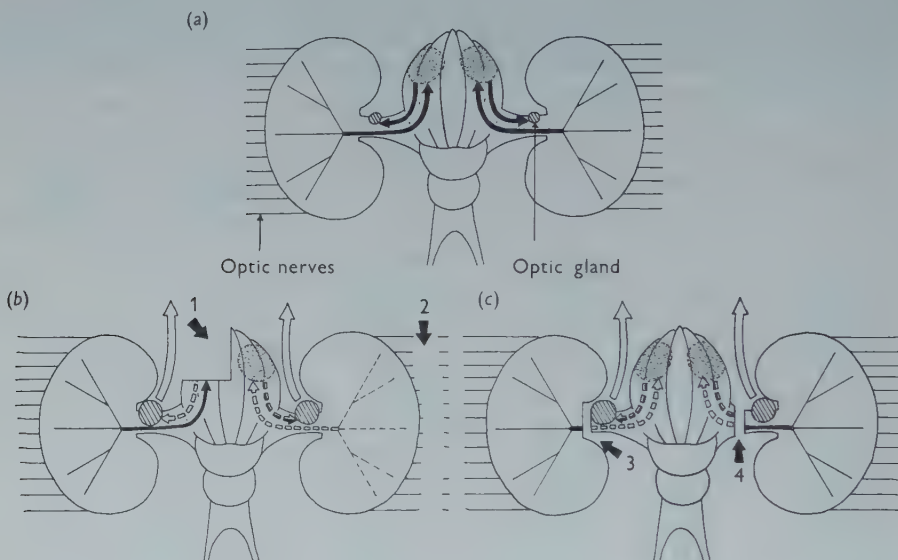
## Males

Reference no.	Category	Gonad wt. (g.)	Body wt. (g.)	Days since operation	Condition of the optic glands
Controls					
B17	—	12.6	1250	—	—
B20	—	17.6	1450	—	—
B134	—	15.4	1290	—	—
B137	—	6.4	1060	—	—
'F'	—	18.4	2020	—	—
'X'	—	31.5	4050	—	—
Experimentals					
A168	2	16.7	1430	71	—
A185	2	11.1	1100	52	—
B3	3*	14.5	1020	63	—
B16	3	16.9	1040	35 28B	—
B21	3*	29.1	1010	73 63B	—
B22	3*	19.1	1120	65 55B	—
B23	3*	13.1	1030	65 55B	—
B52	3*	22.6	1440	8	—
B179	2	9.8	1010	31	—
C1	2	25.4	1510	54	r L
C2	2	20.3	1350	54	RL
C26	3*	23.5	1320	67B 21	RL
C49	3*	12.9	1050	33	RL
C51	3*	16.5	1070	37	r L
C77	3*	10.9	1020	26	RL
C117	3*	15.8	1170	32	R (LHS removed)

## Females

Reference no.	Category	Gonad wt. (g.)	Body wt. (g.)	Ratio	Days since operation	Condition of the optic glands
Controls						
B12	—	3.9	1070	0.0036	—	—
B135	—	2.7	1230	0.0022	—	—
B141	—	2.7	1140	0.0024	—	—
B142	—	2.1	1050	0.0020	—	—
C101	—	2.4	1010	0.0024	—	—
'E'	—	3.6	1120	0.0032	—	—
'Y'	—	6.5	1170	0.0056	—	—
'Z'	—	45.2	2000	0.0225	—	—
Experimentals						
B19	3	4.2	1030	0.0041	35 28B	—
C10	3*	19.1	1230	0.0155	59	r L
C11	3*	17.3	1050	0.0165	58	RL
C14	2	69.1	1220	0.0566	28	RL

lesions including the subpedunculate lobe cause enlargement of the optic glands and gonad and that the rate of this enlargement is greater than the rate of enlargement following blinding alone.



Text-fig. 13. The mechanism of hormonal control of gonad maturation in *Octopus*. (a) Situation in an immature, unoperated *Octopus*, where secretion by the optic glands is held in check by an inhibitory nerve supply, (b) Two operations that cause the optic glands to secrete a product causing the gonad to enlarge, being (1) removal of the source of the inhibitory nerve supply, and (2) optic nerve section. (c) Further operations having the same effect upon the gonads, thus eliminating the possibility that there is also an excitatory innervation, being (3) optic lobe removal and (4) optic tract section.

#### DISCUSSION

Evidence has been presented to show that maturation of the gonad in *Octopus* is controlled by a secretion produced in the optic glands and that these glands are in turn controlled by an inhibitory nerve supply. Interference with this nerve supply in various ways (Text-fig. 13) causes the optic glands (or gland, since all treatments can be carried out unilaterally, affecting only the gland on the operated side) to become swollen with secretion and this is followed by enlargement of the gonad; females may lay viable eggs which they brood in an entirely normal manner. Blinding by optic nerve section or optic lobe removal, while not interfering directly with the innervation of the optic glands (Text-fig. 13), also induces enlargement of the glands and gonads, and again the treatment can be shown to be effective if carried out unilaterally. None of these means of inducing gonad enlargement is effective if the optic glands have first been removed.

The optic glands are innervated by nerves arising in the subpedunculate/dorsal basal region on either side of the posterior part of the supraoesophageal lobes of the brain. This innervation inhibits secretion and does not appear to be paralleled (as

might be expected, see below) by a balancing excitatory innervation, so that blinding must have its effect via the supraoesophageal centres by reducing the restraint on secretion.

Although there is at present no direct experimental evidence available, the effect of blinding upon the state of the gonad implies that the optic gland system will ultimately prove to be governed by changes in photoperiod, in a manner analogous to the regulation of sexual maturity by the pituitary system in vertebrates (Harris, 1955; for a more general review of the effects of changes in photoperiod on animals see Hendricks, 1956).

Optic glands have been found in all cephalopods (except *Nautilus*\*) so far examined, so that there is reason to believe that the mechanism here described from *Octopus* will prove to be general for the group. It has obvious analogies with hormonal systems controlling maturation of the gonads in other animals. The vertebrate pituitary, the insect corpus allatum, and the X-organ—sinus gland complex in the eyestalks of crustaceans all have a similar anatomical relation to the nervous system (Hanström, 1947), and while this anatomical comparison cannot be carried very far because the relevant structures in the higher centres of vertebrates, arthropods and cephalopods have evolved independently within the groups concerned and cannot be homologized, there are close functional parallels between the neuro-glandular systems regulating sexual maturity in the three groups.

All the glands concerned are regulated directly by the highest centres of the nervous system and all serve in one way or another to delay the onset of sexual maturity. The anterior pituitary of vertebrates produces a gonadotrophic hormone under control of a centre in the hypothalamus. The ultimate link in this control appears to be hormonal via the portal blood system of the pituitary stalk; if this is interrupted the gonads atrophy, as they do following lesions to the hypothalamus itself. This however is evidently not the whole story since limited lesions to certain regions of the hypothalamus, produced by tumours, may lead (at any rate in man) to precocious production of hormone and an early onset of sexual maturity, presumably by 'damaging structures that normally inhibit the release of pituitary secretion' (Harris, 1955). The situation in insects appears essentially similar, once metamorphosis has taken place. The corpus allatum, which in larval insects produces a 'juvenile hormone' (Wigglesworth, 1940) inhibiting production of adult characters, becomes inactive shortly before the last moult and later emerges as a source of a gonadotrophic hormone under control of an inhibitory centre in the supraoesophageal ganglion and an opposing excitatory centre in the suboesophageal ganglion (Englemann, 1957).

It is less easy to compare the above three systems with that found in the decapod crustaceans; operations so far carried out in the latter have tended to produce widespread metabolic effects making it difficult to ascertain whether induced changes to the gonads are incidental to, or the direct result of, specific interference with gonad-regulating hormones. It does, however, seem clear that some sort of

\* Prof. J. Z. Young reports (personal communication) that from a preliminary study of a series of sections of *Nautilus* he has been unable to find conspicuous optic glands.

double control exists; removal of the eyestalks causes the gonads to ripen and it appears that this can be attributed to removal of an inhibitory factor produced in the X-organ-sinus gland system (Knowles & Carlisle, 1956), while removal of the Y-organ from the antennary or maxillary segments\* is followed by atrophy of the gonad (Arvy, Echali r & Gabe, 1956). This would imply that the state of the gonad in crustaceans is regulated by a balance between two hormonal products, rather than by changes in the concentration of a single product as would appear to be the case in vertebrates, insects and cephalopods.

Notwithstanding these evident differences the crustacean system clearly falls into the same general category as the others in as much as it too is closely linked with the highest centres of the central nervous system. It seems improbable that this association has occurred independently in the four groups (three of them widely separated) by chance. A possible explanation can be derived from the observation that all these forms in which these control systems have been found are relatively advanced members of the groups to which they belong, being characterized by final forms having a considerable complexity of structure. This is particularly true of the structure of their nervous systems. Vertebrates and cephalopods are remarkable for the extent to which the higher parts of the C.N.S. are developed and both depend to a considerable extent upon learned rather than innate responses; it seems reasonable to suppose that the flexibility of behaviour that this allows is largely responsible for the success of the two groups. Systems that involve learning from experience, however, imply a relatively long post-embryonic development if the animal is to gain advantage from a full development of its potentialities (Wells, 1958*a*), and this necessarily involves a delayed sexual maturity, particularly if, as in cephalopods, the animal starts its free life with those parts of its nervous system most intimately concerned with learning relatively undeveloped (Wells, 1958*b*).

The situation in arthropods, though similar, is complicated by their mode of development. In the decapod crustaceans and insects, where regulating devices have been found these are always associated with development by stages specialized for distribution or feeding; delayed sexual maturity is here desirable because it permits greater, and to a considerable extent independent specialization of both larva and adult (for a discussion of some of these specializations see Hardy, 1956). Once again, however, the final adult form is characterised by a considerable nervous complexity that gives it properties not found in the developmental stages; properties that again would be of no selective advantage to an animal breeding before they became fully developed.

We have now, therefore, four groups in which systems preventing early development of the gonad are closely associated with the presence of final stages having relatively complex structure and behaviour. Comparable systems have not been reported from the less advanced members of the same groups and, while it would be unwise to assert on this basis that such systems are lacking, it is tempting to

\* The location of the Y-organ depends upon the location of the principal excretory organs, being in the antennal segment when the maxillary is excretory and vice versa. The Y-organ is innervated from the suboesophageal ganglion.

speculate that this is because special mechanisms for delaying the onset of sexual maturity only become important where the gonad must be held back until the higher centres of the C.N.S. develop their full potentialities. If this is true it is not altogether surprising to find that the control of the glands responsible comes from these higher centres themselves, and the extraordinary parallelism of the mechanisms for controlling sexual maturity in three such widely separated phyla as the molluscs, arthropods and chordates becomes comprehensible and, indeed, inherently the most probable result of a parallel evolution of complex from more simple forms.

#### SUMMARY

1. Gonad and body weight records have been collected for 487 individual octopuses over a period of 3 years.

2. In the size range used for experiments (200–1000 g.), the ovary is always immature in control animals, and constitutes only about 1/500th of the total body weight. The testis is rather larger, generally ripe, and forms about 1/100th of the body weight, increasing somewhat relative to the size of the body over the range considered.

3. Following optic nerve section, or removal of certain parts of the supraoesophageal lobes, the gonads enlarge. In females the ovary enlarges from 1/500th to as much as 1/5th of the total body weight within 5 weeks of operation and this may be followed by laying of fertile eggs that are brooded in a normal manner. In males where the testis is generally mature before operation the enlargement produced is only of the order of 50%.

4. Enlargement of testis or ovary is always accompanied by enlargement of one or both of the optic glands, and operational treatments that normally cause enlargement of the gonad are ineffective if the optic glands are first removed.

5. The optic glands are innervated from the subpedunculate/dorsal basal area in the hind part of the supraoesophageal brain mass. Lesions in this area, or severance of the nerve tracts running along the sides of the brain from it to the optic glands, cause these and the gonads to enlarge.

6. Unilateral central lesions or optic nerve section cause optic gland enlargement on the operated side only, but appear to be as effective in determining gonad enlargement as bilateral treatments.

7. It is concluded that maturation of the gonad is determined by secretion from the optic glands which is normally held in check by an inhibitory nerve supply from the subpedunculate/dorsal basal lobe area. The action of this region is in turn dependent upon the integrity of the optic nerves and thus, presumably, upon light.

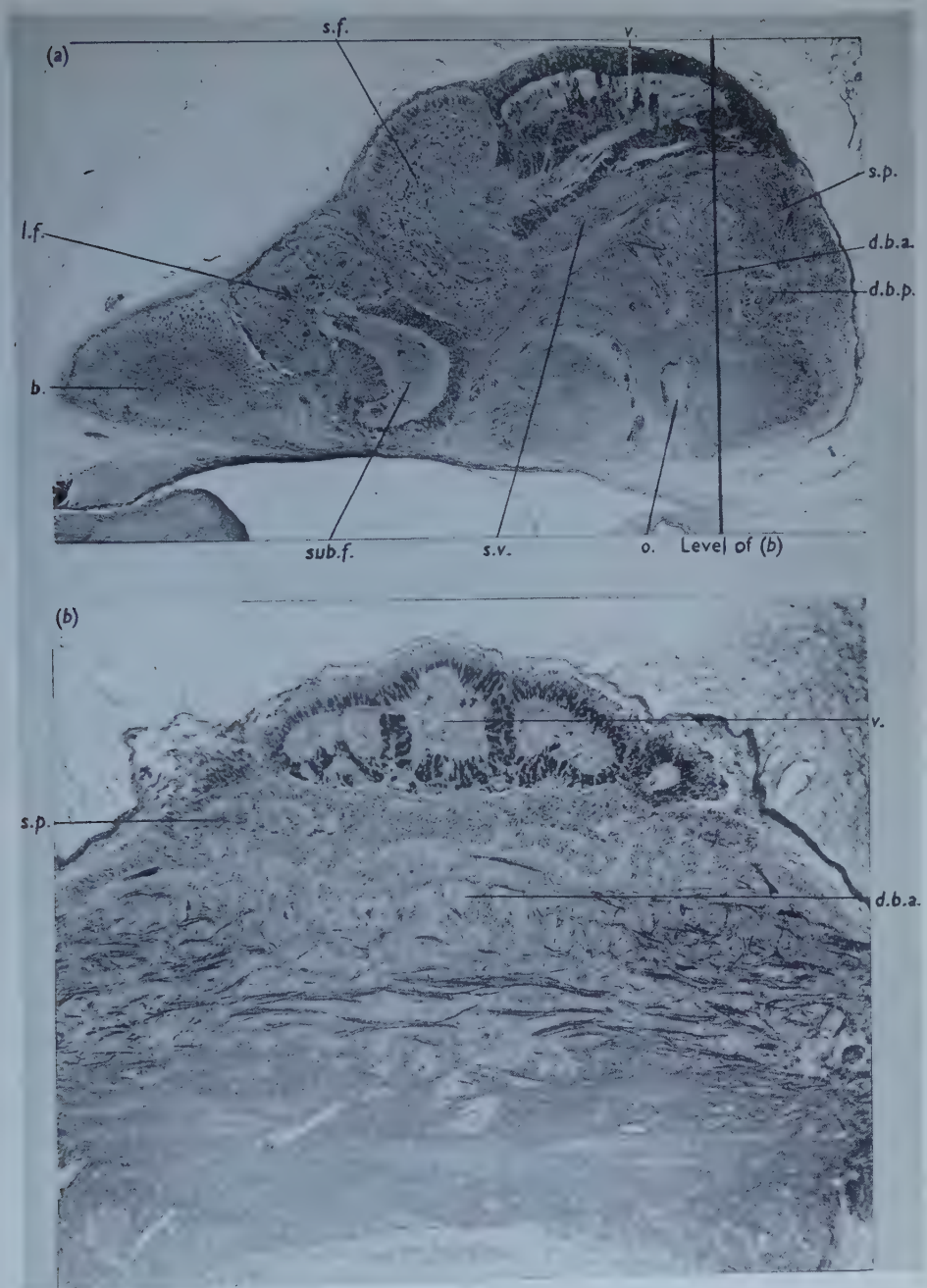
8. This system in cephalopods is compared with analogous systems regulating sexual maturity in arthropods and vertebrates.

We would like to thank Mr B. B. Boycott and Prof. J. Z. Young, F.R.S., for permission to use data collected from their experimental animals and for placing at our disposal sections of the brains of these animals; the photographs for Pl. 1 *a*, *b*, and 2 *b* were taken by Mr J. Armstrong from their material. Prof. Young and

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M. J. WELLS AND J. WELLS—HORMONAL CONTROL OF SEXUAL MATURITY  
IN *OCTOPUS*

(Facing p. 32)



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## EXPLANATION OF PLATES

### PLATE 1

- (*a*) Longitudinal vertical section through the supraoesophageal lobes of the brain of *Octopus*. (*b*) Transverse section at the level indicated on *a*. The lobes of the brain are indicated as follows: *b*, buccal; *i.f.*, inferior frontal; *s.f.*, superior frontal; *sub.f.*, subfrontal; *v.*, vertical; *s.v.*, subvertical; *s.p.*, subpedunculate; *d.b.a.* and *d.b.p.*, dorsal basal anterior and posterior respectively. *o*, optic commissure. Cajal silver preparations.

### PLATE 2

- (*a*) Copulation in *Octopus vulgaris* Lamarck. Male on the left, with his hectocotylized third right arm in the mantle cavity of the female on the right. (*b*) Degenerating nerves in the *LHS* optic gland of octopus *♂QC* 48 hr. after a central lesion (Text-fig. 11) was made. Cajal silver preparation.

## SEX BEHAVIOUR AND SEX DETERMINATION IN *CREPIDULA FORNICATA* L.

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### INTRODUCTION

The problem of sex determination in relation to the formation of chains in *Crepidula fornicata* L. was first raised by Orton (1909) and was further dealt with by him in a series of papers of which the last is Orton (1952). The same problem has also been studied on other species of the same genus, e.g. on *C. plana* by Gould (1952 and earlier papers) and by Coe (1953 and earlier papers); and on *C. aculeata* and *C. walshi* by Hitoshi Ishiki (1936, 1938). The present investigations were directed towards (i) the determination of the normal sex ratio in chains, (ii) the possibility of inducing changes of sex by artificial means and (iii) the cytology of the gonads.

### THE NORMAL SEX RATIO

Statistical analysis was based upon the examination of five separate groups, containing 15, 47, 24, 19 and 18 chains. These included single, double, triple and quadruple chains, ranging from 7 to 19 individuals per chain. Taking all groups together there were: 122 basal empty shells, 404 mature females without trace of penis, 81 'transient' individuals in the sexually inactive condition, 739 mature and immature males with normal penis. 'Visiting' males, not definitely attached to chains, were not taken into account. On a percentage basis there were 39% mature females + empty shells, 6% 'transients' and 55% normal males.

On further examination it was found that in 42 chains, in which the 'transient' condition of individuals in the middle was less obvious than usual, males were directly followed by females. Although the gonads of the first female and the last male were not examined histologically it is reasonable to suppose that they were not in full development and it is certain that copulation between them could not have taken place, the distance being too great. The majority of chains show one or two individuals in the 'transient' state, and in view of the range of variation encountered it seems likely that the complete absence of 'transient' individuals from a chain is not of any significance.

Double, triple and quadruple chains were encountered more frequently in these experiments than in Nature. This may be due to originally single chains having been broken during manipulation and having joined up with other chains in an irregular manner. The side chains, no matter how small a number of individuals they may be composed, provided only that the individuals are of different sizes,

invariably include both sexes. This suggests that sex determination is related to size, all small animals being males and all large animals being females, with representatives of both sexes at intermediate size. In the intermediate size-range it often happens that the normal male/female sequence along the chain is locally reversed, and from this it follows that the sex-differentiating factors cannot be dependent upon such external influences as that of position. In addition to the factor of size there must be some internal factor, possibly genetic or hormonal, as yet undisclosed.

#### ATTEMPTS TO ALTER THE CHANGE OF SEX

(i) *By re-association of separated males.* In September 1954, 456 males (131 large, 87 medium, 238 small) were separated and placed in a wire basket attached to a floating car and submerged in the sea off Woods Hole. The cage was taken up in July 1955 and the animals were accounted for as follows: 153 males, 83 females, 210 dead, 10 lost. Of those remaining alive there were 35% females, 5% 'transients' 60% males, figures which are remarkably close to those obtained from natural populations. It is therefore concluded that a population of males isolated in Nature will tend to revert to the normal sex ratio.

(ii) *By temperature changes.* Evidence of the effects of temperature upon sex change in other animals suggests that the effects are noticeable within a few days. Three experiments were carried out on separated males which were kept in running sea water at 28–30° C. and at 10° C.:

(a) 157 males; 29 days at 28–30° C.; no sign of sex changes.

(b) 274 males; 6 weeks at 28–30° C.; 3 small males became females; 1 large male showed reduction of penis.

(c) 50 small males; 24 days at 10° C.\*; 10 survived; no sign of sex change.  
55 large males; 24 days at 10° C.\*; 20 survived; no sign of sex change.

There is thus no evidence for an effect of temperature upon sex change in *Crepidula*.

(iii) *By injection of extracts of the other sex.* This method was suggested to me by Prof. Moenkin. An extract was prepared in the following way. Twenty-five large females were taken and their pallial organs were ground up with fine sand in 20 ml. sea water and centrifuged. The supernatant was boiled for a few minutes (for purposes of sterilization) then cooled to 20° C. Two series of males were chosen for injection: (a) 25 of the largest size and (b) 22 of smaller size but with distinct penis. Of (a) 6 survived for 23 days without sign of sex change. Of (b) 9 survived for 23 days and one specimen underwent reduction of the penis; it should be remembered, however, that the experiment was carried out in September when reduction and discoloration of the penis is seen in natural populations.

There is therefore no evidence that female sex hormones have any effect in changing the sex of the male.

\* I take this opportunity of drawing attention to a misprint in an earlier paper (Wilczynski, 1935, *Biol. Bull., Woods Hole*, 109, 353): for '0° C.' read '10° C.'.

## CYTOLOGY OF THE GONAD

Cytological observations were made on fresh material of American (Woods Hole) and English origin.

The gonad is an organ with many branches lying mainly on the left of the pallial complex when seen in dorsal view. There is no difference between the sexes in the anatomy of the gonad (Fig. 1).



Fig. 1. *a*, Young male in ventral view; *b*, upper part of male gonad showing the beginning of the vas deferens; *c*, female in ventral view. The branched gonad is shown in black, the hepatopancreas is stippled.

Histologically the gonad has the same appearance (as seen in 'squashed' preparations) throughout all the stages of sex change, male and female sex cells being present together all the time as described by Coe (1936, 1940) for *C. plana*.

Spermatozoa are to be found as free aggregates (Fig. 2*a, b*). A few sperm tails are to be seen attached to the germinal epithelium. These were described by Gould (1917*a, b*) in *C. plana* and he suggested that they were in process of being eliminated by histolysis; but as they are also to be seen in very young males this is unlikely. Spermatozoa of at least five different types have been recognized: (i) a spermatozoon with one or sometimes two tails (Fig. 2*c*) probably representing two

spermatozoa with their heads stuck together; (ii) a long spermatozoon with a small head extending into a long perforatorium (Fig. 2*d, e*); (iii) a spermatozoon with a large thick head (30–50× normal) and a comparatively short thick tail, the perforatorium being shorter than in other forms (Fig. 2*f*); (iv) a rare type, probably a developmental stage of (iii); (v) a spermatozoon which is possibly a modification of an apyren spermatozoon (Fig. 2*h*); it is possible that the structure illustrated in Fig. 2*g* is the mother cell of several apyren spermatozoa as suggested by Gould for *C. plana*.

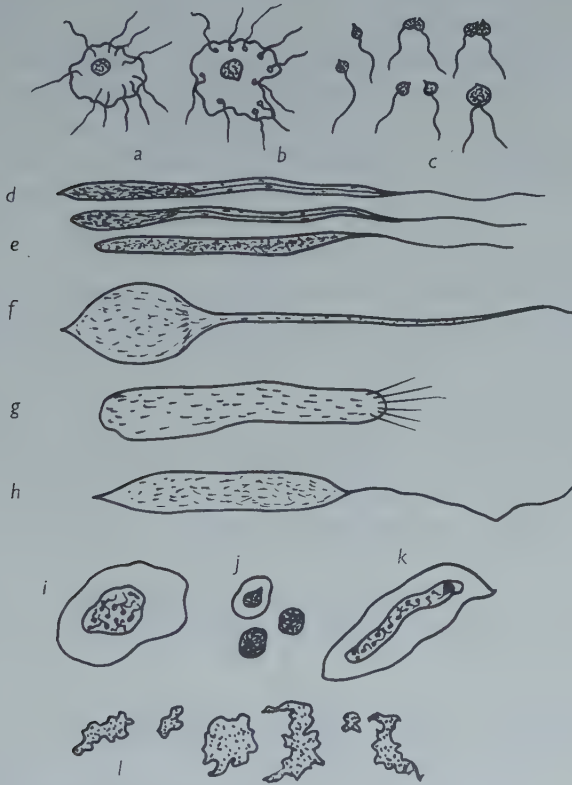


Fig. 2. *a-h*, Spermatozoa and stages in their development; *i-k*, eggs and stages in their development; *l*, yolk cells.

In the same preparations as those in which spermatozoa appear there are to be seen primordial cells, oogonia and oocytes in all stages (Fig. 2*i, j, k*). There are three types of female cell: (i) round, with pale cytoplasm and with a nucleus showing granules and fine chromatin network (Fig. 2*i*); (ii) elongated, with elongated nucleus (Fig. 2*k*); (iii) small with compact nucleus and little cytoplasm (Fig. 2*j*). Besides the oogonia and oocytes mentioned above there are scattered cells of irregular outline containing yellow droplets suggestive of yolk (Fig. 2*l*).

The chromatin in the nucleus is everywhere in the form of granules and of fine interwoven filaments, and it has therefore not been possible to establish the chromosome number.

#### CONCLUSION

Earlier studies on the biology of *Crepidula*, already referred to in the Introduction, together with the results reported in this paper indicate that the chains of *Crepidula* are not breeding associations as was at one time supposed. The distance between males and females in the chain is too great to allow of copulation. Insemination of females must be brought about by small 'visiting' males. It is observed that the seminal vesicles of spawning females are always full of sperm and this suggests that repeated insemination at frequent intervals is unnecessary. The tendency for the normal sex ratio to be re-established among groups of separated males and the regular arrangement in order of size of the individuals forming a chain, together argue that sex determination is a matter of age and/or size. It has yet to be demonstrated that sex determination is susceptible to any other influences.

#### SUMMARY

1. The percentages of males and females in naturally occurring chains of *Crepidula fornicata* L. were found to be 39% females, 6% 'transients' and 55% males.
2. Disconnected males kept in cages in sea water for 9 months re-established the normal sex ratio.
3. Disconnected males were kept for periods of 4-6 weeks at temperatures of 30° and 10° C. No significant tendency to change sex was observed.
4. Males were injected with extracts of females. No tendency to change sex was observed.
5. In animals of all sizes from small males to large females both male and female gametes are invariably present together. The gametes of both sexes are polymorphic.

My thanks are due to Prof. Armstrong, Director of the Marine Biological Laboratory at Woods Hole, Massachusetts, where much of the work was done, and to the American Philosophical Society for a grant during my stay there. I also wish to thank Mr H. A. Cole for sending living specimens by airmail from the Fisheries Laboratory, Burnham-on-Crouch, Essex, to Beirut.

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# STUDIES ON THE TRANSFER OF FERTILIZED MOUSE EGGS TO UTERINE FOSTER-MOTHERS

## II. THE EFFECT OF TRANSFERRING LARGE NUMBERS OF EGGS

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### INTRODUCTION

In the previous paper of this series (McLaren & Michie, 1956) we described an experiment in which fertilized mouse eggs were transferred to the left uterine horn of recipient foster-mothers which were themselves pregnant with eggs of their own. Over the range tested (0-18 eggs), as the number of fertilized eggs transferred to the recipient female was increased, the number of embryos developing from them increased proportionately. We were thus unable to find a limit to the number of mouse embryos which could implant in a single uterine horn. There was, however, a suggestion that post-implantational mortality was greater when the total number of embryos implanted in a single horn was high.

The present work extends the range of inoculum sizes to thirty eggs, in order to investigate further the relation between implantation number and embryonic mortality, and also to determine whether a 'law of diminishing returns' would set in when larger numbers of eggs were transferred.

### MATERIALS AND METHODS

As *donors* we used immature albino females induced to ovulate by hormone treatment and mated to albino males. The albino females were drawn from two random-bred strains. Some came from 'The Mousery', Rayleigh, Essex, and some from the TO (Theiler's Original) strain maintained at the National Institute for Medical Research, Mill Hill, London. The albino males were from the TO strain.

As *recipients* we used female  $F_1$  hybrids between the C3H and C57BL inbred strains mated to albino males of the TO strain. Since the recipients were homozygous for full colour, embryos of donor (alien) and recipient (native) origin could be distinguished at autopsy by their eye colour. All recipient females were adult and had previously given birth to one litter.

Donor females were used  $3\frac{1}{2}$  days after mating, and recipient females  $2\frac{1}{2}$  days after mating. We had previously found this to be the most satisfactory combination.

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Fertilized eggs were transferred to one uterine horn only (the left), so that its contents at autopsy could be assessed against those of the uninjected, control, horn.

Fourteen days after the operation ( $16\frac{1}{2}$  days *post coitum*) the recipients were killed. Each implantation in both horns was classified as 'live alien embryo', 'live native embryo' or 'dead'. The last group was further divided, according to the stage of pregnancy at which death was estimated to have occurred, into late deaths (13–16 days), middle deaths (10–12 days) and early deaths (before 10 days). We have discontinued the use of the terms 'resorbing embryos' and 'resorption rate' since we know of no compelling evidence that dead embryos undergo resorption in the mouse.

Other details of our experimental technique, including details of the egg transfer operation, are given in our earlier paper (McLaren & Michie, 1956).

#### THE PLAN OF THE EXPERIMENT

The recipient females were divided into five groups, as follows:

- (1) Untreated control females.
- (2) Females which received a 'dummy transfer' of saline but no eggs into the left uterine horn.
- (3) Females which received five to ten eggs ('low' group).
- (4) Females which received fifteen to twenty eggs ('middle' group).
- (5) Females which received twenty-five to thirty eggs ('high' group).

The mice were selected for the five groups by a randomizing process which was subject to a restriction designed to spread the collection of data from the five groups more or less evenly over the same period of time.

#### RESULTS

In analysing the pregnancies obtained from the five groups, only surgically satisfactory operations were included. It had previously been found that gross surgical trauma affected embryonic survival. Recipients in which only alien embryos were found at autopsy have also been omitted, since they were presumably pseudo-pregnant, rather than pregnant, at the time of operation.

Table 1. *Pregnancy rate, and average number of implantations*

Group	No. of mice showing vaginal plugs	No. of pregnancies	Average no. of implantations	
			Right horn (control)	Left horn (injected)
Controls	33	25	5.00	3.90
'Dummy'	14	11	4.91	4.73
'Low'	15	13	3.85	5.23
'Middle'	9	9	5.55	8.00
'High'	12	10	6.10	7.20

*Pregnancy rate*

Table 1 shows that the pregnancy rate among the treated females ( $43/50=86\%$ ) did not differ significantly from that of the untreated control females ( $25/33=76\%$ ). This confirms our earlier finding that the operation of egg transfer does not affect the pregnancy rate. The pregnancy rate among control females is significantly higher than the  $57\%$  found in our earlier work. This can be attributed in part to our use on this occasion of  $F_1$  hybrid females known for their high reproductive output, and in part to the improved conditions under which the females were kept prior to mating (in groups of not more than eight mice per cage, as against groups of twenty to thirty).

*Number of implantations*

The reproductive superiority of the recipient females used in the present work over those used previously also manifests itself in the significantly higher number of implantations per horn in the control females ( $4.42 \pm 0.28$  as compared with  $3.39 \pm 0.21$ ). There is no significant correlation between the numbers of implantations in the two horns ( $r = -0.12$ ). Once again the mean numbers of implantations in the right (uninjected) horns of the four groups of treated mice do not differ significantly from the corresponding value in the control females (see Table 1).

The low average number of implantations (3.85) in the right horn of 'low group' females, and the high average number (6.10) in the right horn of 'high group' females, must represent chance fluctuations, apart from the small increment received by the 'high group' from the transmigration of embryos injected into the opposite horn (see Table 3 and McLaren & Michie, 1956). As stated above, females were selected at random for the various groups.

We concluded from our earlier work that the injection of saline alone ('dummy transfers') reduced by about one-third the expected number of implantations in the injected horn. Whether because of the improved quality of our recipient females, or through an improvement in our surgical technique, this effect was not apparent in the present work.

*Yield of live embryos from transferred eggs*

The data summarized in Table 1 were collected over a period of 18 weeks. When the yield of live embryos from transferred eggs is tabulated chronologically for the 'low', 'middle' and 'high' groups (Table 2), there appears to be an improvement of yield with time. A regression analysis confirms the significance of the effect ( $P < 0.05$ ). The calculated regression lines for the three groups do not differ significantly from one another either in slope or in position. Using a common slope, the yields per injected egg adjusted to week 9.44 (i.e. the mean week) are 25.0, 18.9 and 22.2% in the 'low', 'middle' and 'high' groups, respectively. These percentages do not differ significantly from one another, and although the range of egg numbers has been doubled as compared with the previous experiment, there is still no consistent tendency for the yield per injected egg to fall off as the number of eggs injected is increased.

The time-trend, according to the regression line fitted to the three groups, amounts to an increase of about 1.7% per week, rising from 0.07 embryos per egg injected at the beginning of the experiment to 0.36 embryos at the end. This is presumably due to an increase of manual dexterity with time.

Table 2. Yield of live embryos from transferred eggs in the 'low', 'middle' and 'high' groups, arranged chronologically

	Weeks	No. of pregnancies	No. of eggs injected	No. of live aliens	Yield (%)
'Low' group	1-3	5	31	4	13
	6-9	2	14	1	7
	10-13	3	26	12	46
	14-18	3	23	5	22
'Middle' group	1-3	2	34	3	9
	6-9	1	19	5	26
	10-13	2	39	5	13
	14-18	4	75	25	33
'High' group	1-3	2	54	11	20
	6-9	5	130	17	13
	10-13	2	54	13	24
	14-18	1	28	13	46

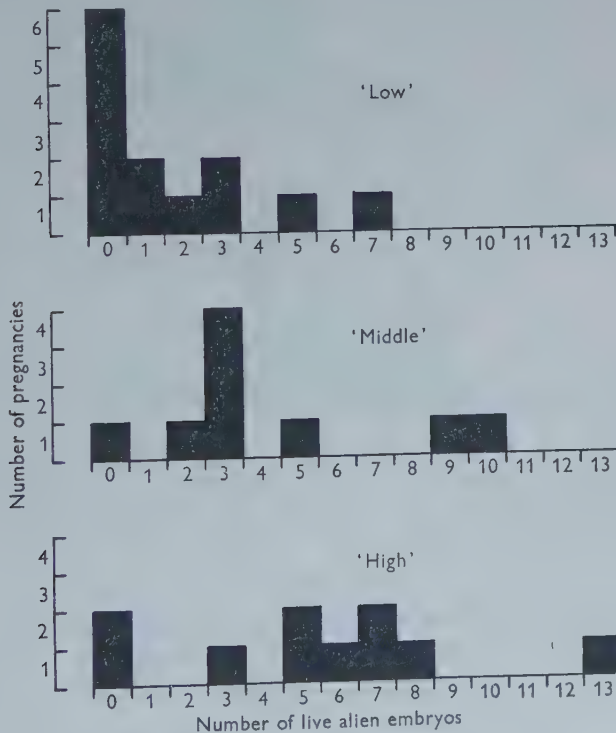


Fig. 1. Frequency distributions of the number of live alien embryos in the 'low', 'middle' and 'high' groups.

Fig. 1 shows the pregnancies in the three groups receiving eggs, arranged according to the yield of alien embryos found in each at autopsy. The class of zero yield is a small one except in the 'low' group, where it can be largely accounted for through the loss of individual eggs. With relatively small inoculum sizes we expect a certain proportion of zero yields simply on the basis of random fluctuation. From our earlier work we concluded that over and above the random loss of individual eggs there was a second and distinct phenomenon of loss of the whole inoculum of eggs as a unit. We estimated that about one-third of all transfers failed through this cause. In the present experiment this hazard has been largely overcome, and accounts for the failure of perhaps 10% of transfers.

*Suppression of native embryos*

(1) *In the injected horn*

Table 3 gives the average number of implantations, classified as 'live alien', 'live native' and 'dead', in the right and left uterine horns of females of the five groups. As the number of eggs injected increases, the number of alien embryos in the injected horn increases, and the number of native embryos in the same horn decreases. A regression analysis indicates that there is a decline of 0.09 native embryos for each additional egg injected ( $P < 0.001$ ). This coefficient is the same as that found in our earlier experiment, but the phenomenon appears more striking in the present experiment as a greater range of inoculum sizes has been used.

Table 3. *Uterine contents of pregnant females in the five groups, at 16½ days post coitum*

Group	No. of pregnancies	Av. no. of eggs injected into left horn	Av. no. of implantations in right horn (control)			Av. no. of implantations in left horn (experimental)		
			Live native	Live alien	Dead	Live native	Live alien	Dead
Controls	25	—	4.40	—	0.52	3.50	—	0.36
'Dummy'	11	0	4.64	—	0.27	3.91	—	0.82
'Low'	13	7.2	3.77	0.00	0.08	2.31	1.69	1.23
'Middle'	9	18.6	5.00	0.11	0.44	2.78	4.11	1.11
'High'	10	26.6	5.00	0.30	0.80	0.80	5.10	1.30

Table 3 shows that in the 'high' group an average of less than one native embryo was found in the injected horn, as compared with five in the opposite horn. It is clear from the Table that the deficit of live native embryos cannot be accounted for from dead embryos visible at 16½ days *post coitum*. We have independent unpublished evidence that embryos dying at a very early stage of pregnancy (*c.* 7 days) are still visible at 16-day autopsy. It follows that the suppression of native embryos by aliens must occur before or during the early stages of implantation and is not a result of post-implantational competition. The alien embryos, being a day ahead of the natives in developmental stage, are likely to implant first, and may render the uterus relatively refractory to the implantation of the native late arrivals.

(2) *In the uninjected horn*

There was significant evidence in our earlier work that when the number of implantations in the injected horn rose above the normal level, the implantation of native embryos in the *uninjected* horn was reduced. But in the new data there is no tendency for the number of implantations in the uninjected horn to decrease as the number of eggs injected increases.

We can therefore say that if the inhibitory effect upon implantation observed in our earlier experiment was a real one, it can only operate where the total number of implantations has been increased to a level approaching the reproductive capacity of the female, and that with our present females we have not reached this level. The frequency distributions of the total number of implantations in the recipient females of the two experiments are given in Fig. 2. The truncated distribution for the first experiment suggests a possible upper limit, or 'ceiling', to the number of implantations. The symmetrical distribution for the second experiment gives no evidence of any such 'ceiling'.

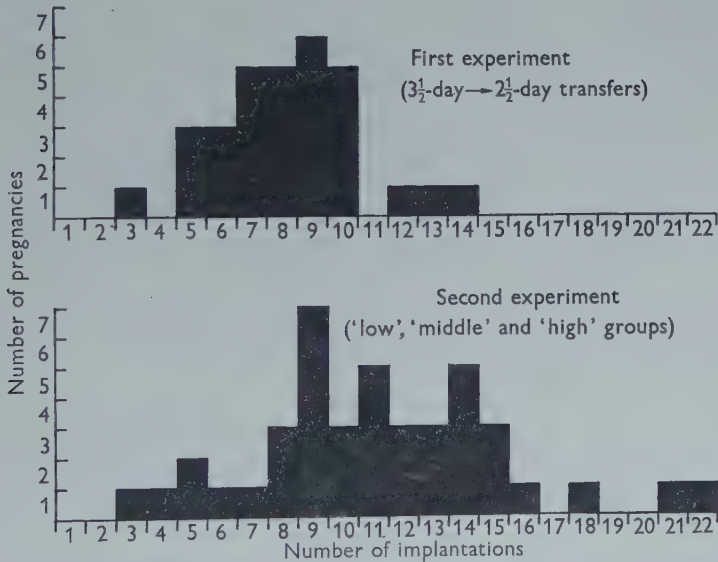


Fig. 2. Frequency distributions for the two experiments of the total number of implantations in recipient females. The upper figure is taken from Fig. 3 of McLaren & Michie (1956) in which the words 'unoperated control females' appeared in the legend in error for 'recipient females'.

*Prenatal mortality*

The data on mortality are given in Table 4. In the control females the prenatal mortality rate was 10%. In contrast to our previous finding there was no significant correlation between the two horns in respect of mortality. This bears witness to the greater uniformity of the females used in the present experiment. Of the twenty-two unsuccessful implantations, eighteen were classified as early deaths, four as middle deaths and none as late deaths.

The mortality in the uninjected horns of treated females did not differ significantly in extent or age distribution from that in the control females. In the injected horns of all groups the mortality rate was about double the control level and averaged 18.2%. The greater frequency of late deaths in the injected horn is not significant ( $P=0.1$  by Fisher's 'exact method').

Table 4. *Prenatal mortality*

	No. of horns	Total no. of implantations	Deaths				Mortality rate (%)
			Early	Middle	Late	Total	
Control females	50	221	18	4	0	22	10
Uninjected horns of experimental females	43	205	14	1	1	16	8
Total for uninjected horns	93	426	32	5	1	38	8.9
Injected horns of females in 'Dummy' group	11	52	7	1	1	9	17
'Low' group	13	68	11	4	1	16	24
'Middle' group	9	72	9	0	1	10	14
'High' group	10	72	9	1	3	13	18
Total for injected horns	43	264	36	6	6	48	18.2

Table 5. *Number of embryonic deaths in the left (experimental) horns of females receiving eggs, arranged according to degree of crowding of the horn*

'Low' group			'Middle' group			'High' group		
No. of implantations	No. of embryonic deaths		No. of implantations	No. of embryonic deaths		No. of implantations	No. of embryonic deaths	
	Early	Middle + late		Early	Middle + late		Early	Middle + late
0	—	—	4	1	0	1	0	1
2	1	0	4	1	0	2	0	0
2	1	0	6	1	0	6	0	0
3	1	0	8	0	0	6	2	1
3	1	0	8	1	0	7	1	1
5	1	3	8	2	0	8	0	0
6	0	0	9	2	0	8	0	0
6	0	1	11	0	0	8	2	0
7	1	0	14	1	1	10	2	0
7	1	1	—	—	—	16	2	1
8	1	0	—	—	—	—	—	—
8	2	0	—	—	—	—	—	—
11	1	0	—	—	—	—	—	—

Table 5 gives the mortality in each injected horn of the females receiving eggs, arranged according to the number of implantations in the horn. In our earlier experiment there was a significant tendency towards an increased mortality rate in the more crowded horns. No such effect is apparent in the present experiment, in spite of the increase in the degree of crowding. This finding is again in accord with the superior reproductive capacity of the females used in the second experiment.

## DISCUSSION

*Technique of egg transfer*

Although the *average* yield of embryos per egg transferred was no higher in the present than in the previous experiment (just over 20%) the improvement with time during the present experiment meant that by the end we were achieving a yield of about 35%. This is comparable with the 40.5% yield achieved by Gates (1956), but not with the 50% yield which in our previous paper we concluded could be attained with our technique, given better control of technical and natural hazards.

The fact that our performance showed such marked improvement in the course of the present experiment, in spite of our having previously carried out some hundreds of similar egg transfer operations, suggest that the technique is difficult to standardize and requires modification before it can be recommended for general use.

*Reproductive limits*

The main aim of the present experiment was to investigate the reproductive limits of the female mouse, which we appeared to be nearing in our earlier experiment. But the  $F_1$  hybrid mice used as recipients on the second occasion were so reproductively competent that in spite of doubling the effective range of the number of eggs injected we failed to detect any limits. Table 6 and Fig. 3 summarize the results of the two experiments.

It was not to be expected under the conditions of our experiment that a limit would be set by the capacity of the single injected uterine horn to accommodate the

Table 6. *Comparison between the first and the second experiment*

	First experiment	Second experiment
Characteristics of control females:		
Number of implantations per horn	3.4	4.5
Embryonic mortality	16 %	10 %
Correlation between mortality in the two horns (evidence of heterogeneity)	Positive	None
Pregnancy rate	57 %	76 %
Technique:		
Proportion of eggs lost as whole inocula	33 %	10 %
Effect of injection as such	Reduced implantation number	Increased mortality
Number of eggs injected	0-18	0-30
Effects of egg transfer on:		
Number of aliens	Increases steadily with number of eggs transferred. No limit found	Increases steadily with number of eggs transferred. No limit found
Number of natives in injected horn	Decreases with number of eggs transferred	Decreases with number of eggs transferred
Number of natives in opposite horn	Decreases when large numbers of eggs are transferred	No effect
Mortality in injected horn	Increases when number of implantations becomes high	No effect

larger numbers of embryos. Hollander & Strong (1950) showed that unilaterally ovariectomized females gestate the normal number of live embryos per pregnancy: since in such pregnancies one uterine horn is empty, the average number of embryos gestated in the other horn is doubled. Even the number of eggs transferred in our 'high' group was not quite sufficient to double the expected number of implantations in the injected horn.

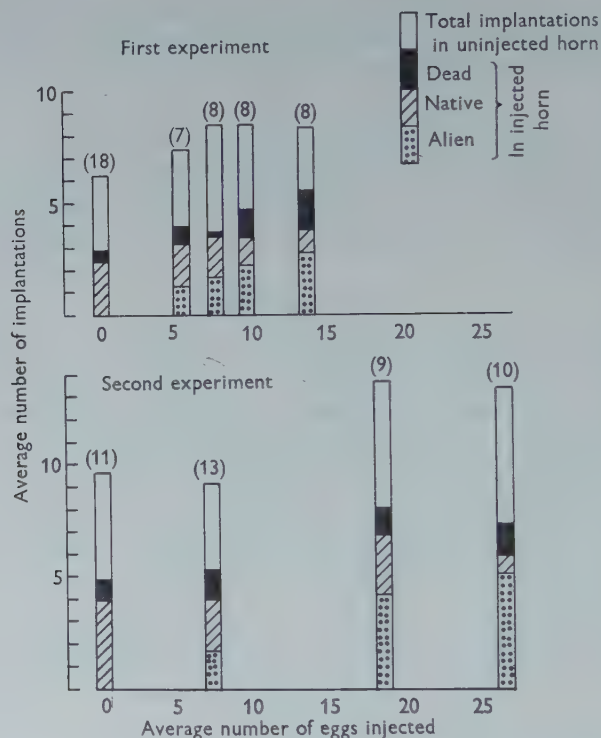


Fig. 3. The results of transferring different numbers of eggs. The upper figure is taken from Fig. 2 of McLaren & Michie (1956). The number in brackets above each column represents the number of pregnancies on which the averages are based.

On the other hand, Bowman & Roberts (1958) have recently obtained results which are difficult to reconcile either with Hollander & Strong's or with our own. These authors counted the corpora lutea and implantations in sixty-six normal pregnancies, and found that the proportion of fertilized eggs represented by implantations in a given uterine horn was inversely related to the number of corpora lutea in the ovary on that side. A similar relation held between the number of corpora lutea and the number of live embryos found at 18½ days *post coitum*, i.e. the more eggs shed, the greater the proportion lost.

The data which Bowman & Roberts have published place the occurrence of this phenomenon in their material beyond reasonable doubt. Yet if a similar process had occurred in Hollander & Strong's experiment, a substantial reduction in mean

litter size should have been observed in their unilaterally spayed females. In the same way we would expect our own experiment to have shown a decline in the percentage yield of alien embryos with increasing numbers of eggs injected. But in fact, as we have seen, the yields obtained in the 'low', 'middle' and 'high' groups were approximately the same. The tendency which we found for the number of *native* implantations to diminish with increasing numbers of eggs injected is not strictly relevant to the effect described by Bowman & Roberts, which relates entirely to interaction between embryos at the same developmental stage as each other.

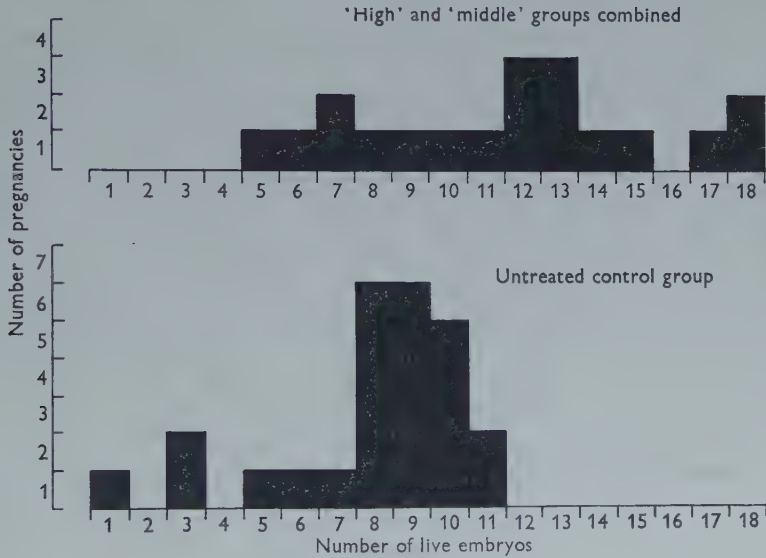


Fig. 4. Frequency distributions of the number of live embryos in control pregnancies and in the 'high' and 'middle' groups.

Leaving the question of single-horn limits, we may turn to the capacity of the female mouse *as a whole* to implant large numbers of embryos. Here we might have expected to find a limit. Yet in our material it proved possible to increase by about 50% the average number of implantations per pregnant female (i.e. from about 9 in the control and 'dummy' groups to about  $13\frac{1}{2}$  in the 'middle' and 'high' groups) without provoking either a raised embryonic death rate or a lowered percentage yield of live embryos from the transferred eggs. The distribution of the number of live embryos in the 'middle' and 'high' groups was thus extended far beyond the control range, as shown in Fig. 4. This is surprising, since (in contrast to the situation studied by Hollander & Strong) the number of implantations must certainly have been raised above the number of corpora lutea available for their maintenance.

## SUMMARY

1. Post mortem examinations were made at  $16\frac{1}{2}$  days *post coitum* of the uterine contents of female mice belonging to the following five groups: untreated control females; females receiving 'dummy' transfers of saline without eggs; females receiving five to ten fertilized eggs ('low' group); females receiving fifteen to twenty eggs ('middle' group); females receiving twenty-five to thirty eggs ('high' group). All transfers were made into the left uterine horn  $2\frac{1}{2}$  days after mating the recipient to a fertile male. Genetic markers enabled embryos of donor and recipient origin to be distinguished by eye colour.

2. The transfer operation did not affect the pregnancy rate, nor the implantation rate in the uninjected horn.

3. The yield of live embryos of donor origin showed a systematic improvement in all three groups throughout the 18 weeks of the experiment, rising from about 7% of eggs transferred at the beginning to about 36% at the end.

4. The percentage yield was not affected by the number of eggs transferred.

5. The implantation of transferred eggs was found to inhibit the implantation of native eggs in the same horn, but not in the opposite horn.

6. Embryonic mortality in the injected horn was approximately doubled by the transfer operation, but was unaffected by the number of eggs transferred.

7. These findings are discussed and compared with results reported in an earlier paper (McLaren & Michie, 1956).

We are grateful to the Agricultural Research Council for financial support.

## ADDENDUM

We have recently (McLaren & Michie, 1959) approached the problem by a different method: instead of increasing the number of implantations by the addition of alien eggs, the native quota was approximately doubled by the administration of gonadotrophins. In this way a limit was found above which foetal mortality sharply increased. This increase was restricted to deaths occurring after day 9, and only those embryos which survived the early period contributed to the effective crowding of the uterine horn. The females used were nulliparous, and foetal mortality rose when the number of implantations in a single horn exceeded eight.

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## TWO COMPONENTS FROM THE COMPOUND EYE OF THE CRAYFISH\*

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### INTRODUCTION

In recent years the electrical response from the crustacean compound eye was observed by Ruck & Jahn (1954) from *Ligia* and by Hanaoka and his co-workers (Hanaoka, 1950*a, b*; Hanaoka *et al.* 1957) from the crayfish. In the previous paper Naka & Kuwabara (1956) recorded the electroretinogram of the crayfish from the corneal surface of the eye and found that the e.r.g. consisted of two components which they referred to as H-I and H-II. H-I responded only to the 'on' of illumination, while the amplitude of H-II was maintained during the stimulus. The present paper deals with the two components separated by means of the microelectrode.

### MATERIALS AND METHODS

Materials used were the crayfish, *Procambarus clarkii*,† which were collected in the field nearby and maintained in the laboratory. Before the experiments the eye was detached from the body. Two methods of preparation were employed. In one series of experiments the compound eye was bisected through the axis of the eye. In the other series a small hole was made at the centre of the corneal surface so as to insert the microelectrode vertically into the receptor layer. Both types of preparation were so fixed in a Ringer pool with vaseline that only the basal part of the eye-stalk was in contact with the Ringer, an indifferent lead being taken from the Ringer pool. Harreveld's physiological solution for crayfish was used throughout this experiment as the Ringer (Harreveld, 1936). A capillary electrode filled with 3M-KCl with a tip diameter of less than  $0.5\ \mu$  was positioned and inserted into the preparation by means of a micromanipulator. The vertical movement of the electrode was measured by a micrometer attached to the manipulator. The potential was picked up by a 954 type tube that served as an electrometer preamplifier (Elmore & Sands, 1949), which was followed by a two-stage d.c. amplifier. The other apparatus was as described elsewhere (Naka & Kuwabara, 1956). The experiments were carried out at room temperature ranging from  $10^{\circ}$  to  $15^{\circ}$  C. Under these experimental conditions a normal response (i.e. an e.r.g. consisting of two components)

\* The contribution from the Department of Biology, Faculty of Science, Kyushu University, No. 70. A part of this work was briefly reported by Kuwabara & Naka (1957*a*). This investigation was partly supported by the Science Research Fund of the Ministry of Education.

† *Cambarus clarkii* in the previous paper.

was recorded for about 1 hr., but this 'normal time' varied greatly from preparation to preparation suggesting that minute damage to the preparation during the operation might have had great effect on its condition.

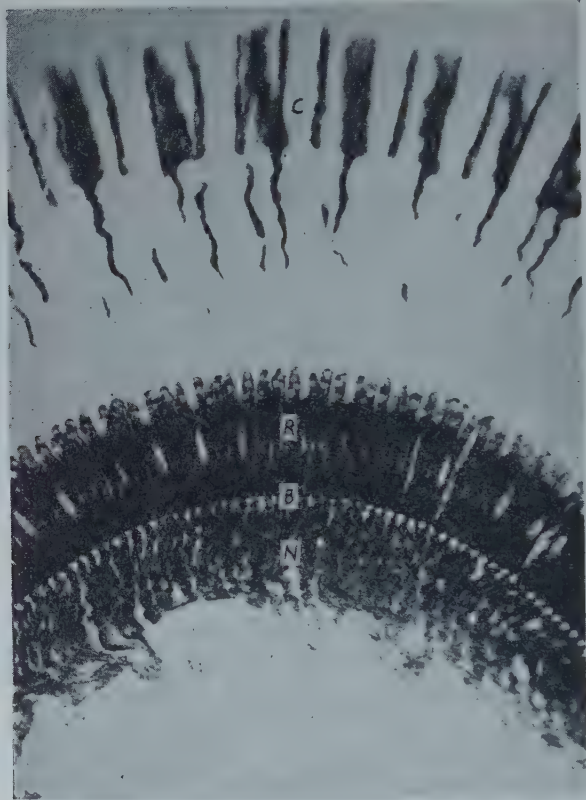


Fig. 1. The micro-photograph of a part of the compound eye of *Procambarus*. C, crystalline cone layer; R, receptor layer; B, basement membrane; N, nerve fibre.

The compound eye of the crayfish consists of three layers: the crystalline cone layer, the receptor layer, and the layer proximal to the basement membrane as shown in Fig. 1.\* The crystalline cone layer is a layer between the cornea and the receptor layer. The receptor layer can also be divided into two parts, the inner and outer parts. The outer part corresponds to the distal part of the retinula cell and the inner part to the basal part of the cell. The inner part also contains the rhabdome, which is surrounded by seven retinula cells. The region proximal to the basement membrane consists of the proximal process of the retinula cell (i.e. the nerve fibre) which proceeds toward the optic ganglion.

\* This section was prepared by Mr T. Samuta. We appreciate his kindness in permitting the use of his preparation.

## RESULTS

*Electroretinogram recorded from the bisected compound eye*

The electrode was inserted into four layers of the bisected eye: the crystalline cone layer, the outer and inner part of the receptor layer and the region proximal to the basement membrane. Three e.r.g.'s were recorded at each layer using light stimuli of three different durations.

The e.r.g. recorded by the electrode inserted into the crystalline cone layer was a negative wave consisting of a negative peak, followed by a slow plateau (Fig. 2).

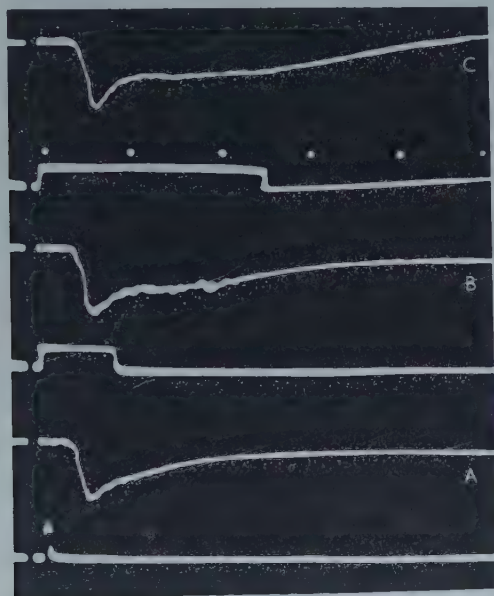


Fig. 2. Electroretinograms from the electrode inserted into the crystalline cone layer. Duration of light stimulus: A, 10 msec.; B, 140 msec.; C, 430 msec. Time,  $\frac{1}{8}$  sec. Reference voltage as in Fig. 3.

According to Naka & Kuwabara (1956) the initial peak was due to H-I which responded only to the 'on' of the light stimulus and the slow plateau was due to H-II which was maintained during stimulation. In Fig. 2 the slow potential became smaller in amplitude with a shorter duration of the flash of light, while the amplitude of the initial potential remained the same.

From the outer part of the receptor layer a diphasic e.r.g. with an initial positive spike-like potential and a following slow negative potential were recorded (Fig. 3). The amplitude and duration of the positive spike-like potential were independent of the duration of the flash of light, but the slow potential increased in both amplitude and duration when the duration of the light stimulus was increased. The positive potential had the same features as H-I with the exception of the polarity of the potential. It was concluded: (1) that the positive potential corre-

sponded to H-I, whose polarity was reversed at this part of the receptor layer; and (2) that the negative potential which responded to both 'on' and 'off' of the light stimulus corresponded to H-II.



Fig. 3. Electroretinograms from the outer part of the receptor layer. Durations of light stimuli are the same as in Fig. 2. Reference voltage, 10 mV.

The inner part of the receptor layer was distinct because of its white guanin deposit. The e.r.g. recorded from this part consisted of only a spike-like positive potential which had the same wave form as that of the positive potential in the diphasic e.r.g. from the outer part of the receptor layer (Fig. 4). The e.r.g. responded only to the 'on' of the light stimulus and the duration and amplitude of the potential was independent of the duration of the light stimulus. It was therefore concluded that the e.r.g. from the inner part of the receptor layer was composed of only H-I, with H-II not being recorded from this part of the receptor layer.

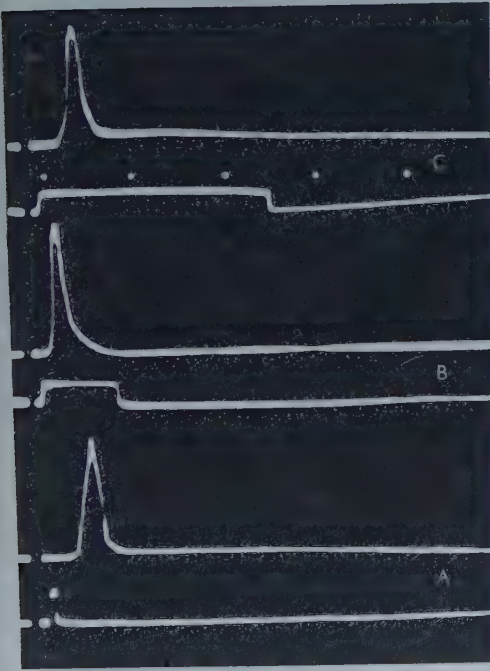


Fig. 4

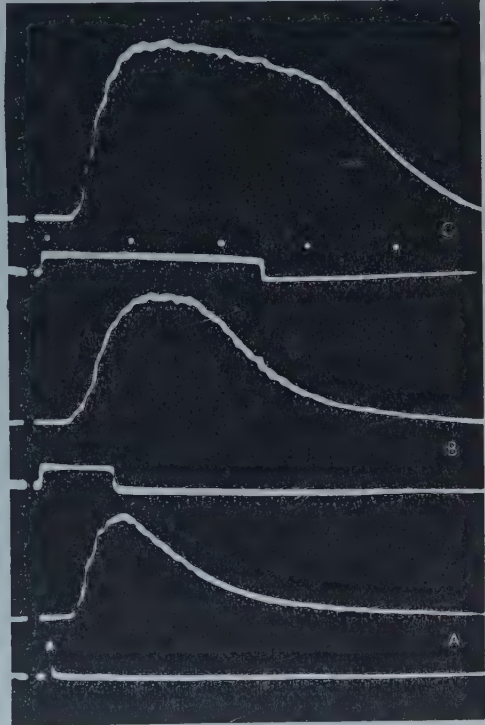


Fig. 5

Fig. 4. Electrophysiological traces from the inner part of the receptor layer. Durations of light stimuli are the same as in Fig. 2. Reference voltage as in Fig. 3.

Fig. 5. Electrophysiological traces from the region proximal to the basement membrane. Durations of light stimuli are the same as in Fig. 2. Reference voltage as in Fig. 3.

The e.r.g. from the region proximal to the basement membrane was a slow positive potential which increased in both amplitude and duration when the light stimulus was increased in duration (Fig. 5).

The wave form of the potential was symmetrical with the slow negative phase of the diphasic e.r.g. from the outer part of the receptor layer, suggesting that the potential corresponded to H-II with an inverted polarity.

*Electroretinogram from the electrode inserted vertically into the eye*

The central portion of the cornea of an excised eye was cut off with scissors to make a small hole through which the electrode was inserted vertically into the eye. The preparation was fixed in the Ringer pool and the axis of the electrode was made to coincide as closely as possible with that of the eye.

When the electrode touched the surface of the preparation, the beam of the oscilloscope was adjusted to the zero level.

The e.r.g. recorded at the position of the electrode, where it touched the surface of the material, was identical with the e.r.g. from the crystalline cone layer and also with the e.r.g. recorded from the whole eye (Fig. 6A).

As the electrode was then moved toward the receptor layer the movement of the electrode did not at first produce any change in the form of the e.r.g., though the response increased in amplitude. When the electrode was inserted deeper there appeared an initial positive potential in the e.r.g. which had the same wave form as that from the outer part of the receptor layer, with the initial positive potential corresponding to H-I and the slow negative potential to H-II. The diphasic e.r.g. was recorded for about 50–100  $\mu$  in depth. If the electrode was inserted deeper the e.r.g. was suddenly transformed into a positive spike-like potential which was recorded for 50–150  $\mu$  in depth (Fig. 6C). Apparently this potential corresponded to H-I and the electrode seemed to be in the inner part of the receptor layer. Even at this depth a slow potential which seemed to be a remnant of H-II was recorded, though of very small amplitude. If the electrode was near the outer part of the receptor layer the slow potential was negative in sign, and if the electrode was near the basement membrane the potential was positive in sign.

The further insertion of the electrode produced a sudden change in the steady potential, and the resting potential became 10–50 mV negative as if the electrode had gone through a membrane. This membrane seemed to be the basement membrane, because there was no other membranous structure which might account for this sudden change in the resting potential. With the change in the resting potential there appeared a positive sustained potential which had approximately the same wave form as the e.r.g. from the region proximal to the basement membrane (Fig. 6D).

From the basement membrane to the region corresponding to the optic ganglion the resting potential changed irregularly, ranging from –50 to –10 mV., and the e.r.g. recorded from this region was composed mainly of the slow potential, H-II, which became smaller in amplitude as the electrode was inserted deeper.

The distance of the electrode from the basement membrane was obtained by assuming that the change in the resting potential occurred when the electrode went through the basement membrane. According to this recalculation, the e.r.g. composed of only H-I was recorded when the electrode was 0–150  $\mu$  from the basement membrane, while the diphasic e.r.g. composed of both H-I and H-II was recorded when the electrode was 150–200  $\mu$  from the basement membrane.

The e.r.g. from the inner surface of the basement membrane usually had the

wave form shown in Figs. 5 or 6D, which seemed to consist of H-II or both H-I and H-II. Direct evidence for this assumption is shown in Fig. 7, which shows three e.r.g.'s recorded with stimuli of different durations, with the electrode in the same position as in Fig. 6D.

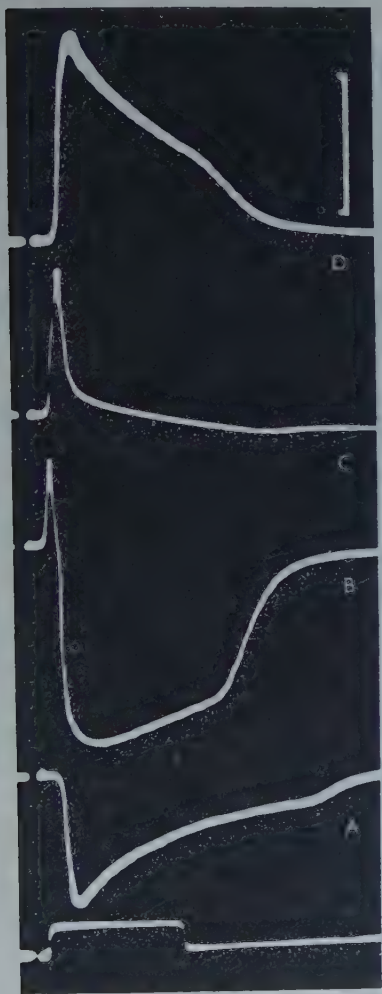


Fig 6

Fig. 6. Electroretinograms recorded from the electrode inserted into different depths of the compound eye. Between the records c and d the resting potential went 35 mV. to negative. Duration of light stimulus 250 msec. Reference voltage, 5 mV.

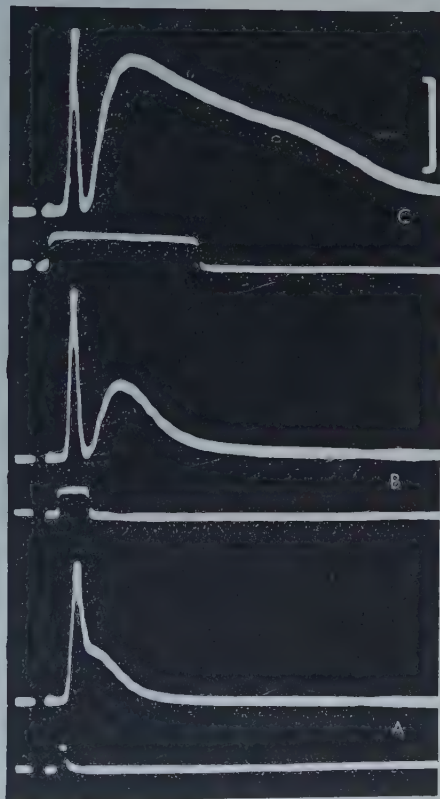


Fig 7

Fig. 7. Electroretinograms recorded with different durations of light stimuli. Electroretinograms were recorded at the same position of the electrode as d in Fig. 6. Duration of light stimulus: A, 10 msec.; B, 50 msec.; C, 230 msec. Reference voltage, 5 mV.

The amplitude and duration of the spike-like potential, H-I, was independent of the duration of the light stimulus, while that of the slow potential increased when the light stimulus was increased in duration.

*Dark-adaptation*

The electrode was inserted vertically into the receptor layer through a hole made at the centre of the corneal surface until a diphasic e.r.g. was recorded. When a diphasic e.r.g. was recorded the preparation was dark-adapted for an hour without

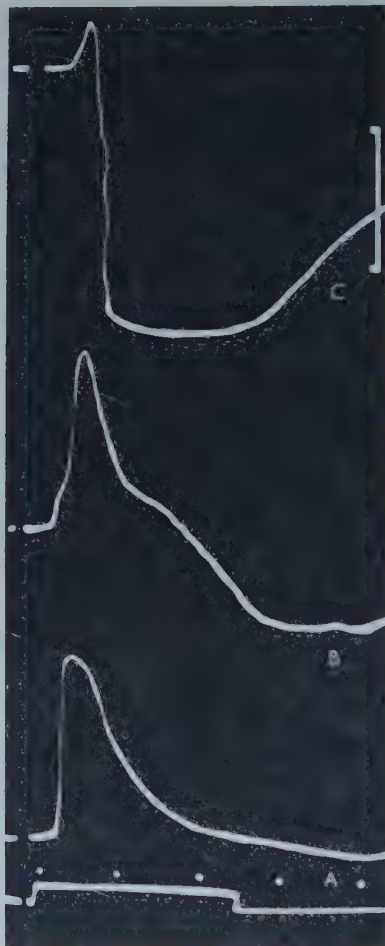


Fig. 8. Change in the wave form of the electroretinograms when the preparation was brought into light-adaptation. A, E.R.G. from a completely dark-adapted eye; B, E.R.G. recorded by the second flash; C, E.R.G. from a moderately light-adapted eye. Three E.R.G.'s were recorded successively. Reference voltage, 5 mV.

changing the position of the electrode. Judging from the wave form of the e.r.g. the electrode seemed in the outer layer of the receptor layer. The response to the first flash is shown in Fig. 8A and is composed of H-I only. The second flash caused the appearance of the negative component, H-II, which indicated that the preparation was brought into a condition of light-adaptation (Fig. 8B).

When the preparation was further light-adapted the negative component, H-II, became larger in amplitude and the e.r.g. became almost negative in sign (Fig. 7c). The decrease in the amplitude of H-I seemed to be caused by light-adaptation and also by interaction with H-II whose latency decreased as a result of light-adaptation.

#### DISCUSSION

The e.r.g. from the crustacean compound eye has been recorded by some authors, but no very extensive study has been made of the subject. The e.r.g. from the compound eye of *Cambarus* has been studied by Hanaoka and his co-workers. According to Hanaoka (1950b) the e.r.g. reversed its polarity at the receptor layer, a diphasic e.r.g. being sometimes recorded from the receptor layer. As to the components in the *Cambarus* e.r.g., Hanaoka & Yasumi (1956) briefly reported that the rhabdome was the site of the slow potential which induced a 'response potential'. Ruck & Jahn (1954) observed polarity reversal of the *Ligia* e.r.g. with leads from the central retina and optic ganglion, and this reversal occurred even in the absence of the ganglion.

In the previous report Naka & Kuwabara (1956) have analysed the e.r.g. from the *Cambarus* compound eye into two components according to the difference in the wave form under light- and dark-adaptation. The two components, which were referred to as H-I and H-II, could only be isolated by means of adaptation because the e.r.g. was recorded from the corneal surface. In the present investigation the two components were separated by the use of the microelectrode.

Some characteristic features of the two components which had been reported in the previous paper, and have been confirmed in the present report, are as follows: H-I responded only to the 'on' of illumination while H-II was maintained during the light stimulus. The amplitude of H-I was not affected by the duration of the light stimulus when the stimulus was longer than 10 msec., whereas H-II increased in amplitude when the duration of illumination was increased. The e.r.g. from the completely dark-adapted eye was composed of H-I alone, while light-adaptation was followed by the appearance of H-II, thus making the e.r.g. the sum of the two components. The change in the wave form of the e.r.g. resulted from the difference in adaptation as is clearly shown in Fig. 8. This phenomenon can be interpreted as a kind of facilitation of H-II.

In this investigation the diphasic e.r.g. was recorded when the electrode was in the outer part of the receptor layer or when the electrode was 150–200  $\mu$  from the basement membrane, and the e.r.g. composed of H-I alone was recorded when the electrode was in the inner part of the receptor layer or when the electrode was 0–150  $\mu$  from the basement membrane. In the *Procambarus* compound eye the length of the retinula cell and that of the rhabdome are about 200  $\mu$  and 150  $\mu$  respectively, the two structures thus differing in length by 50  $\mu$ . The diphasic e.r.g. seemed to be produced as the result of the difference in length of the structures which were responsible to the generation of the two components, H-I and H-II. These considerations lead to the conclusion that the two components originated from different structures in the receptor layer, probably from the retinula cell and from the rhabdome.

The e.r.g. from the insect compound eye was found by Kuwabara & Naka (1957*b*, 1958) and Naka & Kuwabara (1959) to be composed of two monophasic components with opposite polarity. The negative potential from the receptor layer of the insect compound eye was maintained during the illumination and it reversed its polarity at the basement membrane. Under favourable conditions the negative potential was recorded even 1 or 2 days after preparation, while the positive potential was recorded only for 1 or 2 hr. (Naka & Kuwabara, 1959). In *Procambarus* Naka & Kuwabara (1956) reported that the slow potential, H-II, was reproduced for as long as 100 hr., whereas H-I disappeared within a much shorter time.

These similarities between the two potentials, the negative component from the receptor layer of the insect compound eye and H-II, suggest that the two potentials may play the same role in the visual mechanism of the compound eye.

Although the positive components from two kinds of compound eye were both variable, the two potentials differ in wave form. But it seems to be not unlikely that H-I is a primitive form of the positive component in the insect eye because after degeneration the positive component became somewhat like H-I. (Cf. Naka & Kuwabara 1959, fig. 4.)

The polarity reversal of the negative component from the receptor layer at the basement membrane was also observed in *Lucilia* by Naka & Kuwabara (1959). According to Naka & Kuwabara (1959) the reversal of the negative component at the basement membrane was an active process, which meant that the reversed positive potential was induced by the negative potential in the receptor layer. In further support of this assumption Naka (1959) reported on the insect compound eye that the positive potential from the region proximal to the basement membrane was preferentially abolished by the application of KCl solution, while the negative potential from the receptor layer remained unaffected. These results on the insect compound eye suggest that the polarity reversal of H-II at the basement membrane was also an active process and that the slow positive wave from the region proximal to the basement membrane was induced by the slow negative wave, H-II, in the receptor layer.

#### SUMMARY

1. The aim of the present investigation was to confirm the results obtained in the previous paper with the aid of the microelectrode.
2. It is concluded that the two components, H-I and H-II, originate from different structures in the receptor layer.
3. It is shown that a difference in the steady potential ranging from 10 to 50 mV is maintained across the basement membrane.

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## RESPIRATORY REFLEXES IN THE DOGFISH

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The classical experiments of Hering & Breuer (1868) and of Head (1889) demonstrated that inflation of the lungs in mammals resulted in inhibition of inspiration, and that this response was abolished by vagotomy. The Hering-Breuer reflex subsequently came to be regarded as of crucial importance in the generation of respiratory rhythms. The vagal inhibitory inflow, augmented by that from a supra-medullary brain-stem centre, was believed to segment the output of a tonically active inspiratory centre into a series of separate respiratory acts (Pitts, Magoun & Ranson, 1939). Although it is now generally believed that the mammalian medulla deprived of both vagal and supra-medullary influences is capable of initiating cyclic breathing, the vagal inflow is still thought to be important in regulating the pattern of respiration (Breckenridge & Hoff, 1950). The possibility that afferent activity initiated by inspiration and carried by the vagus might be concerned in the regulation of branchial respiration in fish has not previously been explored. In this paper the existence of such reflex regulation is reported from experiments on twenty-five dogfish. A brief report of this work has already appeared (Satchell, 1958).

Specimens of the dogfish *Squalus lebruni* (Vaillant) were fixed by a system of rigidly clamped brass pins which screwed into the cartilage in front of the eye and above the auditory capsule. The trunk was similarly fixed at six places along its length. The fish was perfused through the mouth with sea water, cooled sufficiently to maintain the body temperature between 8 and 12° C.

In some preliminary experiments the outputs of two sensitive strain gauges, connected to the spiracle closer and the pharyngeal wall respectively, were fed into separate d.c.-coupled amplifiers and displayed on a double-beam oscilloscope. Since the movement of the pharynx always followed the movement of the spiracle, both in timing and in amplitude, the movement of the spiracle alone was recorded in subsequent experiments and the lower beam was used to monitor inflation. This was effected by an air-filled balloon in the pharynx connected both to a hand bulb, and, by a side arm, to a tambour pressing against the strain gauge. Very brief periods of inflation were produced by an electromagnetic device either manually controlled or triggered from the sweep. Nerve discharges were recorded with external platinum leads and displayed after a.c.-coupled amplification; they were also monitored with a loud-speaker. In one experiment records of single respiratory neuron discharges were made with Ling-Gerard glass micropipettes. Records were taken with a Grass camera (model C.4.D.) on continuously moving film.

*The response to pharyngeal inflation*

A pharyngeal inflation of moderate duration (19–20 sec.) reduced both the rate and amplitude of respirations during the period of inflation. Fig. 1 A–C shows three successive inflations of increasing extent. The response to a small inflation was a reduction in the rate of respiration, but the amplitude was little altered. With larger inflations the individual respirations became increasingly shallow. After deflation, and occasionally after inflation, the fish made one or more expulsive movements of the pharynx in which water was shot out, not only from the gills but also from the mouth and spiracle. This expulsive reflex has been commented upon by several previous workers (references in Brown, 1957). It occurs spontaneously in aquarium fish, and can be evoked by stimulation of any part of the body. It is thought that the reflex serves to rid the gills of foreign matter. This reflex was recorded in the experiments as a deep respiration (Fig. 1 B, C); it induced a further period of inhibition which was succeeded by a period of hyperpnoea with respirations of increased amplitude. Although inflations were frequently terminated by this expulsive effort, it was not always present.

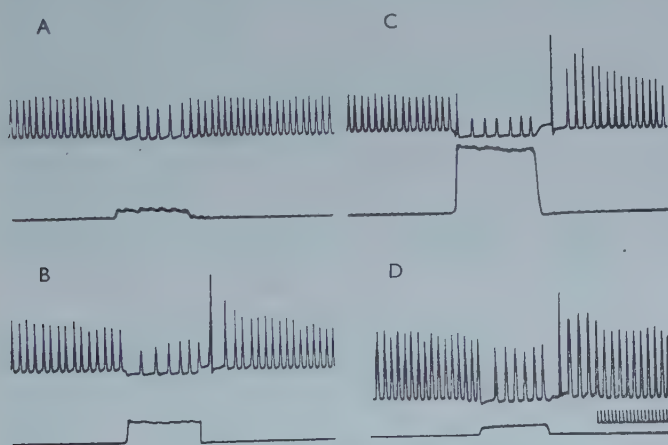


Fig. 1. A–C, the response to successively greater inflations; D, the response to inflation, from another experiment. Top beam: spiracle closer movement; bottom beam: inflation. Time, sec.

A feature noticeable in Fig. 1 A–C, but demonstrated better in Fig. 1 D from a different experiment, is that the inhibition was more obvious at the start and finish of an inflation than it was during the period of inflation. The significance of this will be discussed when the discharges in the vagal afferent fibres are considered.

That the reduction in amplitude of respiration during inflation was not due to faulty recording resulting from skeletal distortion caused by the inflated balloon was demonstrated in two ways. By means of Ling-Gerard type glass micropipettes records of the discharges of respiratory motor neurons were made. It was possible to locate the motor nucleus of the VIIth nerve as some of the cells penetrated could be fired antidromically (i.e. with a latency of 0.5–1 msec.) by stimulating the hypo-

mandibular nerve. Inflations resulted in a reduction in the bursts of discharge of these VIIth nerve motoneurons from 5 to 7 at each inspiration to 2 or 1 or none at all. After deflation they started firing again as before. In other experiments, recordings of the discharge in the branchial branches of the vagus were made from a fine twig dissected free and mounted on recording leads. Here again, during inflation, the bursts of discharges were reduced in duration.

*The response to tetanic stimulation of a branchial branch of the vagus*

A tetanic stimulus (square waves, 7 msec. duration, 15/sec.) applied to the central end of any of the cut branchial nerves caused a more or less complete inhibition of respiration depending on the strength. Fig. 2D, E shows the response to tetanic stimulation of the left 3rd branchial branch of vagus. With a weak stimulus slow respiration started up again after 16 sec.; with a stronger stimulus (Fig. 2E) inhibition was complete and some effect persisted for two or three respirations after the end of the stimulus. As with inflation, a brief period of hyperpnoea supervened after the inhibition.

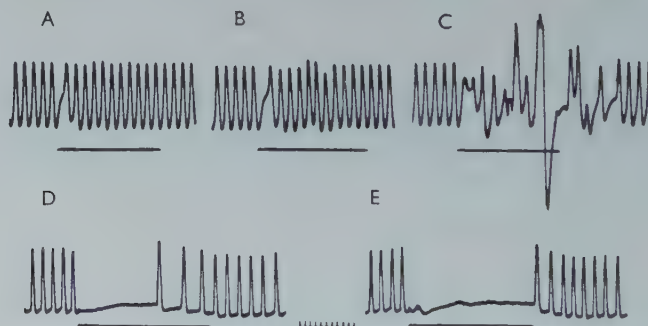


Fig. 2. A-C, the response to tetanic stimulation of the cornea of the eye, with successively stronger shocks; D, E, stimulation of the left 3rd branchial branch of vagus. Top beam; spiracle closer movement; bottom beam; duration of tetanic stimulation. Time, sec.

*Is the inhibition following inflation due to some non-specific stimulation?*

It is well known that in mammals the stimulation of pain fibres may cause either inhibition or acceleration of respiration. In the experiments it was found that any nocuous stimulus to the fish, such as stroking the cornea, pricking, or tetanizing an afferent nerve, also had both inhibitory and acceleratory effects on respiration. In Fig. 2A-C are seen the responses to the tetanic stimulation of the cornea of the left side, with successively stronger shocks. With a weak shock there was a brief period of inhibition at the start of the stimulus. In Fig. 2B this period of inhibition was succeeded by three inspirations that are slightly more rapid than before. In Fig. 2C with a still stronger shock there was a disorganization of respiration with breaths of different size and inhibitory events mixed up with excitatory ones. A comparison of this trace with that in 2D or E, where a branch of the vagus was being stimulated, makes it clear that the inhibition that results from branchial nerve stimulation or

inflation of the pharynx is to be distinguished from that arising from stimulation of other receptive fields. It was never possible to produce the complete and sustained inhibition mediated by the vagus from nerves anatomically unrelated to the respiratory apparatus.

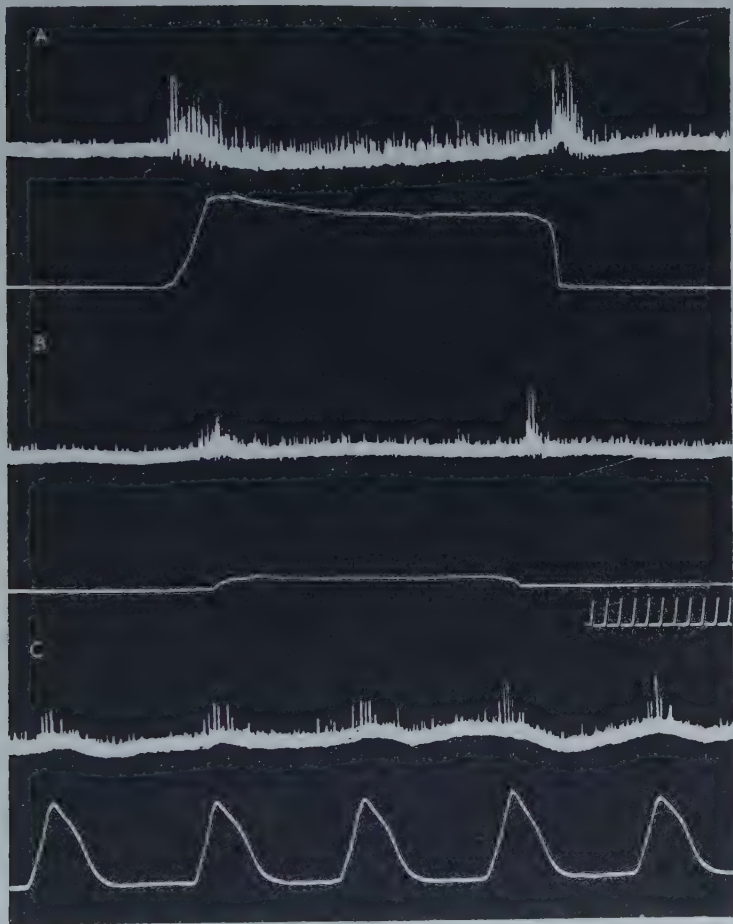


Fig. 3. Action potentials recorded in the distal end of the cut 3rd branchial branch of vagus. Upper beam; A, B and C, action potential records. Lower beam; A and B, inflation; C, spiracle closer movement. Time, A and B,  $\frac{1}{10}$  sec.; C,  $\frac{1}{5}$  sec.

#### *Discharges in vagal afferent fibres*

If inflation produces inhibition by firing pharyngeal receptors which send their afferent fibres along the branchial nerves, then it should be possible to record the discharges of these fibres in the peripheral end of a cut branchial nerve. In such a preparation any activity recorded must originate peripherally and be destined for transmission to the medulla. Fig. 3 A, B shows records obtained from a fine twig split off the main trunk of the peripheral cut end of the left 3rd branchial branch of

the vagus. The responses to two inflations of different extent are illustrated. The water was turned off for the few seconds whilst the record was taken as there are receptors in the pharynx which fire continuously when the water is flowing, and they obscure the response to inflation. That inflation causes receptors to fire is clear; two sorts of discharge can be recognized. There was a firing when the balloon was being inflated and deflated, and a less vigorous sustained firing during the period of inflation. It is probable that one arises from receptors stimulated by movement, and the other more specifically from tension. If the pattern of discharges in Fig. 3 A is compared with the pattern of inhibition in Fig. 1 D a resemblance is seen. The bursts of activity at the moments of inflation and deflation correspond with the more intense inhibition at these times, whilst the sustained activity between could account for the lesser degree of inhibition during the period of inflation.

That these receptors are fired by the normal respiration occurring during an experiment is demonstrated in Fig. 3 C, where the lower beam was used to monitor the spiracular closer. There was a small cluster of action potentials at the apex of each inspiration; their firing also caused rhythmic bursts of sounds on the audio channel, each synchronous with pharyngeal movement. As can be seen in Fig. 4, where both spiracular closure and pharyngeal contraction were recorded simultaneously, the closure of the spiracle preceded the pharyngeal contraction by approximately 0.1 sec. Hence the discharges of the receptors recorded in Fig. 3 C, were really occurring during the active phase of pharyngeal contraction rather than at its peak.

*The dependence of the inhibitory effect on the time of onset of a brief inflation*

By using an electromagnetic inflating device it was possible to inflate the balloon for a period as short as 200 msec. and to 'place' this inflation at any chosen position in the respiratory cycle. From a series of more than 100 such inflations it became clear that they were equally effective in any position during the inter-respiratory pause, but were ineffective or much less effective if they occurred during the rising phase of an inspiration (Fig. 4 A, B). This is interpreted as indicating that the firing of receptors or the occlusion of their afferent pathways at inspiration makes them less able to respond to an artificial inflation at this time. When the pharynx is once more at rest inflation results in additional inhibitory impulses passing up the branchial nerves and so delays the onset of the next inspiration.

*The influence of the activity relayed in branchial afferents on the pattern of respiration*

No differences in the manner and tempo of respiration were detectable by direct observation of fish under aquarium conditions and under experimental conditions. In normal respiration each respiratory movement is followed by a pause (Fig. 5 A). When the anterior cardinal sinus of each side was opened and all the branchial branches of the IXth and Xth nerves cut the pattern promptly changed to that seen in Fig. 5 B. Inspiration followed expiration without intervening pause and the rate of respiration increased. That this change was not simply due to circulatory in-

sufficiency resulting from opening the sinuses was shown in a sham experiment. Both sinuses were opened and the superficial ophthalmic nerves were cut. All the branchial nerves were left intact. Even 3 hr. later the respiratory pattern had not changed, although the rate had slowed slightly. As circulatory failure sets in, the respiration tends to slow down rather than to accelerate, but the inter-respiratory pause remains.

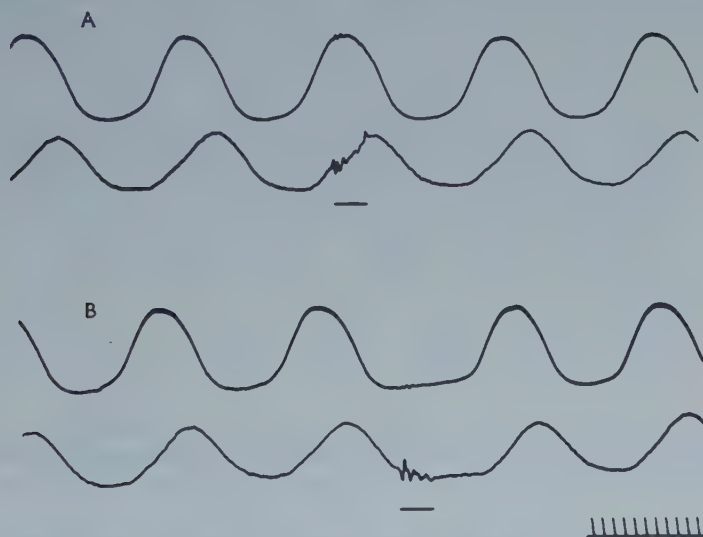


Fig. 4. The response to a brief inflation. Top beam; movement of spiracle closer. Bottom beam; movement of pharynx. The black line indicates the position and duration of the inflation in the respiratory cycle. Time,  $\frac{1}{10}$  sec.

A second method of demonstrating the change in respiration that results when the respiratory centres are deprived of the inhibitory inflow from the branchial nerves was to paralyse the respiratory muscles by a slow injection of tubocurarine, whilst simultaneously recording the contraction of the spiracle closer and the motor discharges in a dissected twig of the vagus. As the curare took effect and the respiratory movements declined in amplitude, the respiratory rate increased. The strain gauge recorded sixteen successively smaller respirations between the onset of curarization and complete paralysis and during this time the respiratory rate increased steadily from 34/min. to 44/min. The stream of sea water supplied the fish with oxygen, and records were still being taken three hours later so the acceleration was unlikely to have been due to anoxia. Curare may exert some specific effect on the cells of the respiratory centre, but the similarity between these results and those of branchial nerve section suggests rather that curare freed the respiratory centres from the inhibitory discharges engendered by respiratory movement.

That the acceleration following section of the IXth and Xth nerves was due to the elimination of most of the inhibitory feed-back was further shown by stimulation of the proximal end of a cut branchial branch. In Fig. 6A and B the restoration of

the inter-respiratory pause by a repeated single stimulus to the proximal end of the 3rd left branchial branch of vagus is shown. It was possible to evoke pauses of varying length depending on the strength of the stimulus to the nerve: it was not found possible to restore the pauses by continuous tetanic stimulation of the vagus, although many combinations of strength and frequency were tried.

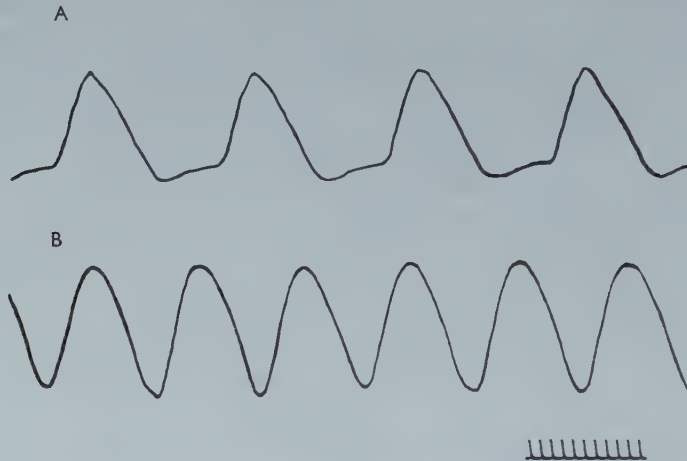


Fig. 5. The change in respiratory pattern and rate resulting from section of the branchial branches of IXth and Xth nerves. A, before section; B, after section. Time,  $\frac{1}{10}$  sec.

#### *The influence of the pre-medullary centres on the respiratory pattern*

In mammals the existence of a pontine facilitatory centre has long been known (Lumsden, 1923). In the dogfish transection between the medulla and the mesencephalon (the pons is vestigial in elasmobranch fish) leads to no marked change in normal respiration. The rate was sometimes a little slower, but the most noticeable result was an increased sensitivity to the effects of pharyngeal inflation. In some fish a moderate inflation would inhibit inspiration completely, a result never seen in fish with an intact brain. There is thus some evidence that centres rostral to the medulla exert an influence antagonistic to vagally relayed inhibition. Brain transection combined with vagotomy resulted in the same pattern of rapid respiration with no pause between breaths such as was seen with vagotomy alone. There was never any development of apneusis as is seen in the mammal when vagotomy is combined with a transection through the anterior pons: if facilitatory centres exist in the mesencephalon, they do not appear to be very potent.

#### *The afferent pathways of the inhibitory reflex*

The contrast between the predominantly inhibitory response elicited by tetanizing a branchial branch of the vagus and the complex intermixture of inhibitory and excitatory responses that results from tetanizing the cornea or an ophthalmic nerve has already been described. Using the response to a tetanic stimulation as a criterion, it has been possible to assess the extent to which the different motor nerves to the

pharynx and jaws carry inhibitory afferent fibres. All the four branchial branches of the vagus and the branchial branches of the IXth nerve produce complete inhibition like that depicted in Fig. 2E. The pre-spiracular branch of the VIIth was also equally potent in inhibition, a result that is of interest in that the spiracle though devoid of gill lamellae in the dogfish is generally considered to be a modified gill slit. The palatine branch of the VIIth was moderately effective, but respiration broke through after 3-4 sec.; no other branch of the VIIth was effective, nor were the maxillary and mandibular branches of the Vth.

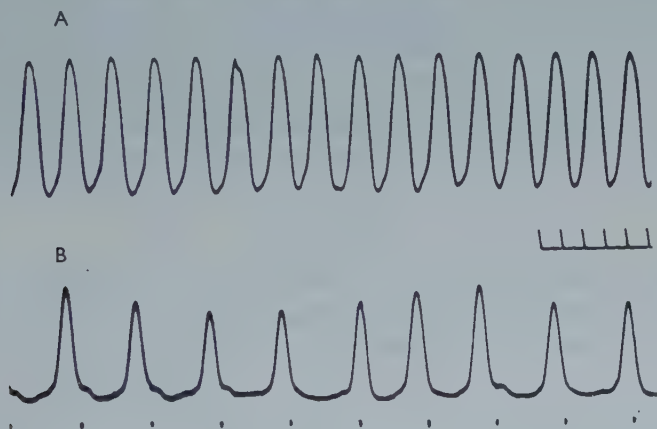


Fig. 6. The effect of a repeated single stimulus to the central end of a branchial branch of vagus when all the branchial branches of IXth and Xth nerves have been cut. A, without stimuli; B, with stimuli. Time, sec. Marks = single stimuli.

### DISCUSSION

The inhibitory reflex described here bears a resemblance to the Hering-Breuer reflex of mammals. In both, inflation excites receptors which discharge up the vagus and inhibit inspiration. Evidence from comparative anatomy and embryology (Goodrich, 1930) is held to point to an origin of the lungs of tetrapods from a posterior pair of gill pouches, and it would seem that a reflex mechanism elaborated to subserve branchial respiration has survived these evolutionary changes and has been utilized in the reflex control of respiration in mammals. That inhibitory afferents in the dogfish run in the branchial branches of the VIIth, IXth and Xth nerves reflects the fact that a fish retains more of the primitive metameric organization of the respiratory system than does a mammal. Since the lungs of tetrapods develop from a single pair of pharyngeal rudiments, it is not surprising that afferent fibres running from them travel only in one pair of nerves.

A further point of interest lies in the response to vagotomy. In the intact or decerebrate mammal this results in a slowing of respiration. The primary respiratory centre responsible for the generation of respiratory rhythms is located in the medulla, and this is played upon by both facilitatory and inhibitory influences from the pontine and mesencephalic reticular formation. Part of the afferent drive

pouring in from the vagus serves to facilitate the inhibitory system, and it is the loss of this after vagotomy that causes the over-facilitation evinced in the deeper, slower, sighing type of respiration (Breckenridge & Hoff, 1950). By contrast, vagotomy in a mammal deprived of these facilitatory and inhibitory influences by a transection between the pons and medulla results in an acceleration of respiration indicating that the vagus exerts a simple inhibitory influence at this level (Kerr, Dunlop, Best & Mullner, 1954). Vagotomy in the intact dogfish caused an acceleration of respiration; the fish responded like a mammal deprived of the regulatory influences of the higher centres. Moreover, transection immediately above the medulla in dogfish had little effect other than to increase the sensitivity to inflation. In their relative independence of down-flowing facilitatory influence the medullary respiratory centres resemble other generators of motor rhythms. The spinal mammal can neither walk nor stand. The spinal dogfish swims incessantly (Steiner, 1885). In locomotion as in respiration the dogfish is less dependent on the facilitatory support of the higher centres.

#### SUMMARY

1. Inflation of the pharynx of a dogfish causes an inhibition of respiration manifested as a reduction in rate and amplitude.
2. Tetanic stimulation of the central end of a cut branchial nerve also inhibits respiration.
3. These inhibitory responses differ in their greater regularity and duration from the transient inhibition arising from stimulation elsewhere in the body.
4. Both normal respiration and inflation cause the discharge of receptors whose impulses pass up the vagus nerve. The pattern of firing of these receptors during an inflation corresponds to the pattern of inhibition.
5. Brief inflations are more effective in securing inhibition if they arrive at a time when the receptors are not being caused to fire by a normal inspiration.
6. Cutting the branchial branches of the IXth and Xth nerves eliminates the pause between successive respirations and increases the respiratory rate.
7. These pauses can be made to reappear by periodically stimulating the central end of a cut branchial nerve.
8. Section of the brain between the medulla and the mesencephalon increases the sensitivity to inflation.
9. Inhibitory afferents run in all branchial branches of the IXth and Xth nerves and in the pre-spiracular branch of the VIIth nerve.
10. It is suggested that in its response to vagotomy the dogfish resembles a medullary mammal.

I would like to express my thanks to Mr V. Hansen, who supplied the dogfish, to Mr E. A. Annand and Mr C. Morris, who designed and made the inflating device, and to Prof. A. K. McIntyre and Dr J. R. Robinson, who discussed the manuscript with me. I am indebted to the Medical Research Council of New Zealand for supporting this work financially.

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## THE NERVES AND MUSCLES OF MEDUSAE

## VI. THE RHYTHM

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Previous papers in this series contained references to the swimming rhythm of medusae, in particular to the temporary change in frequency which can follow stimulation of the subumbrellum. The present paper is a collection of observations on the normal rhythm as determined by a single marginal ganglion, and the varied effects of stimulation thereon. Sufficient data is not yet available for the formulation of a comprehensive theory of the origin and control of the rhythm; however, some of the more readily observable data which bears on this topic can now be given. The term *marginal ganglion* refers to the concentration of nerve cells on the stalk and round the base of the *tentaculocyst*, which is the anatomical term for the whole organ including mesogloea and a terminal mass of crystals. For *Aurelia* the structures were described by Schäfer (1878).

Several features of the rhythm emerge from the work of previous authors. Romanes (1877) established that the rhythm originates in the tentaculocysts, provided strong evidence that it first appears in nervous tissue and found that the frequency is modified by stimulation of distant regions of the bell. In one particular experiment Romanes showed that in *Aurelia* the efferent motor excitation could be initiated in the tentaculocyst by afferent excitation which was accompanied by a wave of contraction of the marginal tentacles. Later workers extended these findings; Horstmann (1934*a, b*) concluded that sensory excitation is converted to motor impulses in the ganglion. Pantin & Vianna Dias (1952) deduced that the pacemaker is within the through-conducting limits of the nervous pathway which co-ordinates the symmetrical contraction at each beat; Horridge (1956*a*) concluded that the ganglion contains a polarized junction at which the diffuse (= *primary* or multipolar) nerve net modulates the frequency of the pacemaker in the giant fibre motor net, but at which excitation does not pass in the reverse direction. Other workers, e.g. Fränkel (1925), Bozler (1926*a, b*) have emphasized that sensory cells within the ganglion region (on and around the stalk of the tentaculocyst) also influence the frequency of the rhythm. However, in all these accounts the character of the rhythm as a time series is not recorded, and the actual modification of the rhythm by artificial means has not been described.

The intervals between the beats of a single isolated ganglion are not uniform and therefore a statistical treatment is necessary. A decision must then be taken as to the treatment of the variability between the eight or more ganglia of one individual

and between individual animals of the same species. Ganglia may show various modes of activity whose separate realities may be demonstrated by statistical tests. Some of these modes are atypical, or known to be the result of stimulation, but those ganglia which fail to respond in a typical way do not invalidate conclusions drawn from the typical mode of activity.

#### METHODS

Some records of the rhythm were made by the standard kymograph technique. The lever was attached to the mesogloea so that the primary nerve net was not mechanically stimulated by the hook or clip. But as a rule neither intact animals nor parts bearing a tentaculocyst beat normally under such circumstances since they are necessarily constrained by pins, stretched or placed in an abnormal orientation. Later observations were made on isolated freely floating segments 2–4 cm. across, each containing a single tentaculocyst, and the intervals were recorded on cash-register paper by a pen relay. A second pen was used in experiments in which a control ganglion was simultaneously recorded. The relay was operated by an observer with a morse tapper key. The key was pressed at the instant when a particular part of the bell passed a readily observable part of its traverse at each contraction; this arrangement ensured that an irregular rhythm did not take the observer by surprise. The method was satisfactory only because the intervals between beats were greater than 3 sec. The observer did not know the expected result of the experiment. Each diagram of this paper shows a different method of plotting the results.

#### THE RHYTHM OF A SINGLE GANGLION

A segment, which is an eighth of a jellyfish and bears a single tentaculocyst, continues to beat for days in a dish of aerated sea water. Several hours after isolation the rhythm becomes free from cyclical changes. This may be checked later by statistical methods, but in practice when the number of beats per minute becomes reasonably constant and cyclical changes are not noticed, the ganglion is tentatively considered suitable for experimentation.

Examples of such a rhythm in *Aurelia* are shown in Figs. 1*a* and 2*a*. A characteristic of the rhythm of a single undisturbed ganglion of *Aurelia*, *Cassiopea*, *Chrysaora*, *Cyanea* and *Pelagia* is that the intervals are far from being of equal duration and the average frequency is less than half the maximum frequency at which the ganglion may discharge when stimulated. The standard deviation of interval is usually 20–30% of the mean; therefore many observations are necessary to establish that differences of mean interval are significant. Previously this point has not been reported.

The rhythm is a time series which can be considered as beginning at any point when a large number of intervals are taken; the first-order serial correlation coefficient ( $r_1$ ) is often significantly less than zero. Figures of  $r_1 = -0.3$  to  $-0.4$  are frequently found: out of ten likely records tested, eight had significantly negative values of  $r_1$  between  $-0.25$  and  $-0.4$ . A negative first-order serial correlation coefficient means that long intervals tend to be followed by short intervals and vice

versa; the correlation is rather low, and to establish a correlation coefficient of  $-0.25$  at the 95 % level sixty intervals must be recorded and 100 at the 99 % level. The second-order serial correlation coefficient ( $r_2$ ) has not been observed to be significantly negative. However, in three cases  $r_2$  was significantly positive when  $r_1$

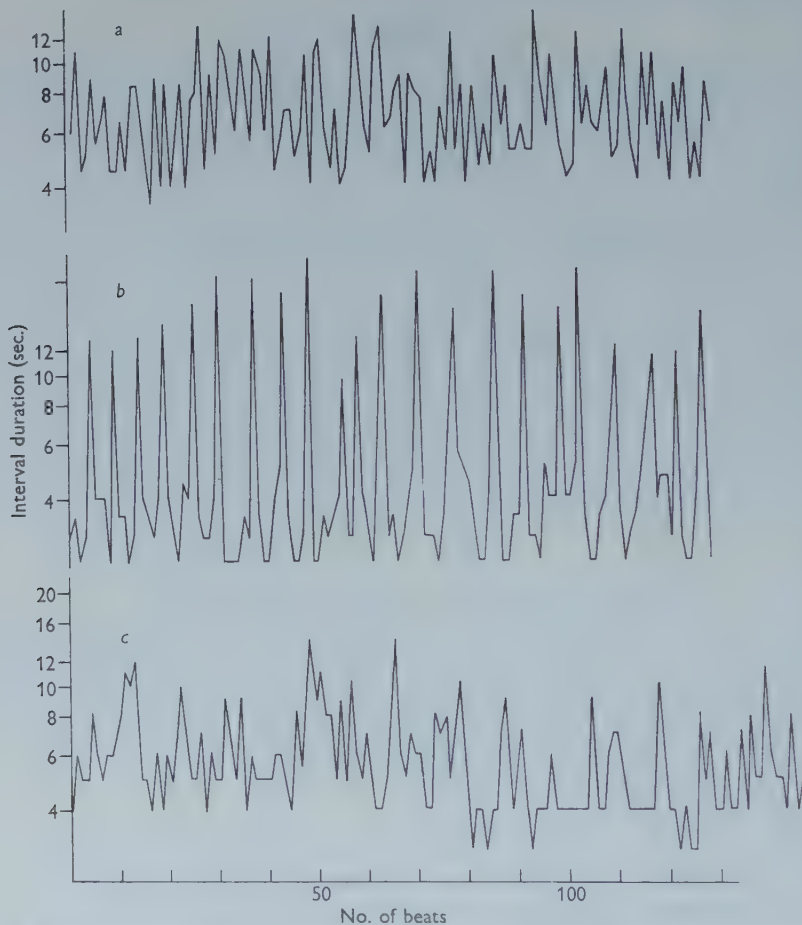


Fig. 1. (a) The normal experimental rhythm of a ganglion of *Aurelia*. The duration of each interval is plotted upwards on a logarithmic scale and the points thus placed at equal distances from left to right are joined by straight lines. Mean interval, 7.1 sec.;  $\sigma$ , 2.51 sec.;  $r_1$ ,  $-0.287$ ;  $N$ , 130. (b) The same ganglion immediately following a mechanical stimulus (a pinch at the bell margin). Long intervals alternate with bursts of shorter intervals. The numerical constants are not given since they are meaningless in such an example. (c) The same after 1 hr. showing a recovery from the cyclical rhythm to a pattern that temporarily has positive first-order serial correlation. Mean interval, 5.8 sec.;  $\sigma$ , 2.5 sec.;  $r_1$ ,  $+0.176$ ;  $N$ , 180.

was significantly negative. A positive second-order coefficient means that the interval is similar to the next but one, whether long or short. For example, a single ganglion of *Aurelia* gave the following figures: number of observations ( $N$ ) 67, mean interval ( $m$ ) 7.68 sec., standard deviation ( $\sigma$ ) 2.9 sec., first-order serial correlation coefficient

( $r_1$ )  $-0.38$ , second-order coefficient ( $r_2$ )  $+0.23$  (significant at 95 % level). The figures for the *Aurelia* ganglion in Fig. 1 (a) are  $N=130$ ,  $m=7.1$ ,  $\sigma=2.51$ ,  $r_1=-0.287$  (significantly less than zero at 99.9 % level),  $r_2=+0.12$  (significantly greater than zero at 90 % level).

The pattern of the rhythm described above appears to be that to which an intact ganglion of *Aurelia* settles down after some hours of isolation. For reasons that will be discussed below, experiments on the rhythm must be made with a single ganglion and not with the whole animal. This rhythm will therefore be called the *normal experimental rhythm* of a single ganglion. This is the optimum state for experiments on the modification of the rhythm, and appears to correspond with the natural rhythm of an intact animal.

Other types of rhythm occur. Commonly a single ganglion attached to a kymograph lever shows a cyclical pattern similar to that of Fig. 1 (b). This is a result of severe mechanical stimulation or of continual stimulation of the diffuse nerve net. Such a rhythm may settle down in a matter of minutes, but sometimes cyclical changes persist for several hours. As the cyclical changes slowly disappear the variability falls and the first-order serial correlation coefficient goes from positive to negative. An example of slow recovery is shown in Fig. 1 (b) and (c).

A regular rapid rhythm at a frequency of 0.3–0.5 per sec. is usually a consequence of stimulation; a preparation attached to a kymograph lever may continue to beat in this way for several hours but if removed from its attachment often slows considerably. The disturbance of the rhythm caused by attaching the mesogloea to the lever may persist for the duration of the experiment. Such preparations may give information of some possible responses of a ganglion but they are not characteristic of the intact animal.

#### STIMULATION OF THE GANGLION VIA THE PRIMARY NERVE NET

Romanes (1877) observed that a temporary acceleration of the rhythm follows the removal of a piece of the bell. He also showed that the ganglion can initiate a beat in response to a distant mechanical stimulus. These observations have been confirmed by later workers.

The normal experimental rhythm of an *Aurelia* ganglion is shown in Fig. 2a, together with ten repetitions following electrical stimulation of the primary nerve net (Fig. 2, b–k). The stimulus was a short burst of twelve shocks at 4 per sec. applied to the tentacular margin of the bell outside the area of the giant fibre net. The stimulus did not produce a contraction wave directly. Intervals of half an hour between the experiments appeared to be sufficient for a full recovery. The diagrams, typical of many experiments, show that the rhythm is accelerated for about ten beats following the stimulus. The initial rapid rhythm then either slows down or is terminated abruptly by a long pause. After this the rhythm is slower and more variable than normal. Fig. 2 contains the whole of the data; some of this information is lost if the results are combined, but the result is then easier to visualize. In Fig. 3 the mean instantaneous frequency of this ganglion is plotted

for each second following the stimulus. The instantaneous frequency is the reciprocal of the interval between consecutive beats. Each point on the graph is a mean of the ten measurements of instantaneous frequency from the data illustrated in Fig. 2. The standard deviations represented by vertical lines are drawn only at 10 sec. intervals. The average frequency of the normal experimental rhythm is shown by the horizontal line, together with its standard deviation based on 70 intervals. Fig. 3 shows the same result as given above in Fig. 2 but most of the unusable data is excluded. At the 95% level of significance points on the right-hand side of the curve show that the rhythm has not recovered its normal average frequency at 90 sec. after the stimulus.

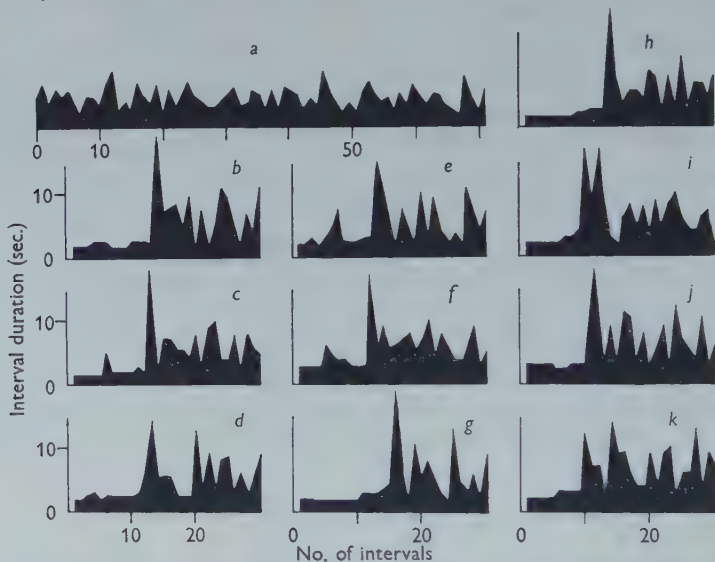


Fig. 2. (a) The normal experimental rhythm of a ganglion of *Aurelia*. The duration of each interval is plotted upwards on a linear scale and the points thus placed at equal intervals from left to right are joined by straight lines. (b) – (k) The rhythm of the same ganglion following an electrical stimulus to the primary nerve net, as described in the text.

The initial acceleration is followed by a definite slowing. The instantaneous frequency 50 sec. after the stimulus is  $0.1 \pm 0.04$  per sec. compared to the normal  $0.24 \pm 0.075$  per sec. and the difference is highly significant (better than 99.9%). Following this slower period the intervals are more variable: calculations from the raw data show that from the fifteenth to the thirtieth beat following the stimulus the standard deviation of the intervals was 7.3 ( $N=150$ ) compared to a normal of 2.35 ( $N=70$ ); the increase is significant (better than 99%). Over the same period, corresponding with the aggregate of right-hand halves of the diagrams (b) to (k) of Fig. 2, the first-order serial correlation coefficient increases from  $-0.21$  to  $-0.41$ , a change which is significant at 95% level. The second halves of the experimental records (b) to (k) subjectively appear more variable than normal and appear to have sharper peaks with more indentations; the calculations show these appearances to be significant.

In contrast to the usual behaviour, a small proportion of the ganglia temporarily slow down following stimulation of the primary net. Some examples of both *Aurelia* and *Cyanea* ganglia consistently showed this effect following a mechanical stimulus at a distance from the ganglion. The ephyra larvae at once stop beating if one arm touches a food particle and do not beat during the course of the maintained contraction of an arm in the feeding response. *Nausithöe* and many Hydro-medusae behave similarly. The ganglia of adult *Aurelia* and *Cyanea* which behave in this way are therefore not outstanding exceptions, though they appear to be uncommon.

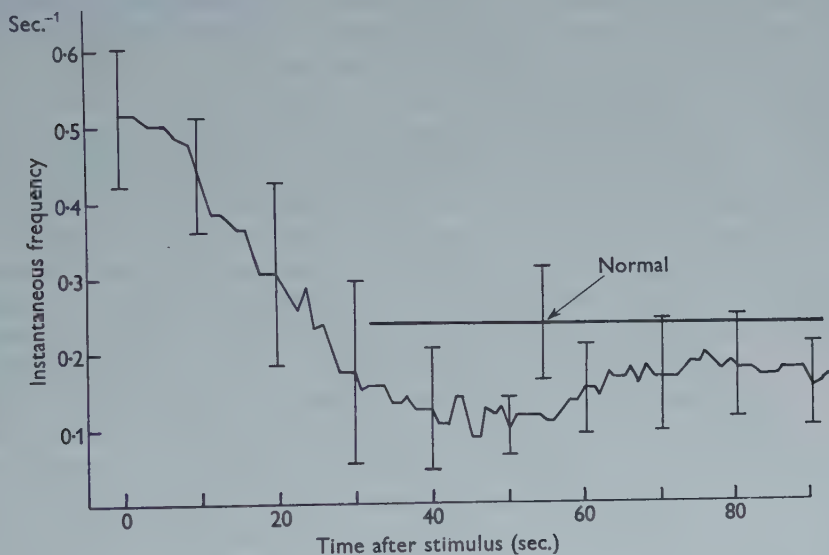


Fig. 3. The average change in frequency with time following an excitation of the primary nerve net. This figure is derived from the data of Fig. 2 (*b-k*) by calculating the average frequency over the ten experiments at each second following the stimulus. The vertical lines represent standard deviations for each 10 sec. The previously observed mean is also shown together with its standard deviation based on 70 intervals, as in Fig. 2 (*a*).

Repeated stimulation of a ganglion often produces a rhythm in which relatively long intervals are interspersed with bursts of rapid beating as in Fig. 1(*b*). This cyclical rhythm reverts to a more normal state after a few hours unless the ganglion is damaged. It bears no relation to the fishing behaviour of *Gonionemus* (Agassiz, 1862), which periodically inverts and sinks from the surface without beating. I have not observed alternate bursts and long pauses in intact Scyphozoa in the sea.

## STIMULATION VIA THE GIANT FIBRE NET

### (*a*) Previous work

The traditional view, common to almost all workers, of the origin of the rhythm of medusae is that a state of excitation builds up to a threshold at which the pacemaker fires off an impulse. Such a rhythmical system is described as a relaxation

oscillator: this continues to be an acceptable hypothesis because no likely alternative theory has been suggested. The only experimental data so far reported which has any bearing on this theory is the effect of an extra beat artificially introduced into the rhythm.

Working with *Rhizostoma*, Bethe (1903) described the interval which follows the extra beat as longer than normal, suggested that the longer interval sometimes *compensated* for the previous shorter one, and compared the scyphozoan rhythm with that of the frog heart. However, the compensatory pause of the frog heart appears because there is always an impulse in transit in the fibres which conduct towards the ventricle. An artificial stimulus to the ventricle produces an antidromic impulse which cancels with a spontaneous impulse and never reaches the place of origin of the rhythm in the pacemaker. After an extra beat the rhythm of the heart is therefore in step with its former rhythm.

Pantin & Vianna Dias (1952) re-examined the compensatory pause of *Aurelia* and made two new observations: (a) that the stimulus which produces an extra beat may cause a change in the frequency, and (b) that the interval which follows an intercalated beat is usually about as long as a normal interval. They conclude that the compensatory pause is not truly present in medusae but that it sometimes appears accidentally when there is an appropriate change in frequency. The second observation, (b) above, agrees with a theory that the basis of the rhythm is a relaxation oscillator in which a pacemaker repeatedly charges to a threshold and discharges again. An antidromic impulse would then discharge the ganglion from whatever level it had reached and would be followed by an interval of normal duration if no other factors enter into the situation. This is described as a resetting of the rhythm, and according to Pantin & Vianna Dias is the usual outcome of this experiment in *Aurelia*. If an artificially induced extra beat can reset the rhythm the impulses of the normal rhythm of the most rapid ganglion presumably also reset the rhythms of the other ganglia.

Experiments with one or more antidromic impulses (at intervals of 2 sec.) have now been repeated. Ganglia which are discharging rapidly and regularly usually show only a reset of the rhythm at the same frequency as before, i.e. the interval following the extra beat is similar to the other intervals. This confirms Pantin & Vianna Dias; however, such preparations are in my experience stimulated to saturation via the diffuse nerve net or they would not beat so rapidly. Further stimulation of the primary nerve net or of its continuation in the ganglion has little effect; an antidromic impulse is then followed by a pause of the usual length. Such preparations are readily observed in kymograph experiments; an example is shown for *Cassiopea andromeda* (Fig. 4a, b), and *contra* Bethe (1903) the same effects are usually observed in *Rhizostoma* (Ross, private communication). In these circumstances the origin of the rhythm is within the through-conducting giant fibre net. The argument for this conclusion is fully set out by Pantin & Vianna Dias (1952). However, different results which do not agree with the theory of a simple reset of a pacemaker are also found, at least in *Aurelia*, *Cassiopea* and *Cyanea*.

(b) *An apparent compensatory pause*

A single ganglion of *Cyanea* was observed continuously for 4 hr. for most of which time it beat with an interval of  $3.0 \pm 0.25$  sec. Experience with other preparations indicates that the persistence of this rapid rhythm was probably in part a consequence of periodic experimental stimulation. During the 4 hr. sixty observations of the effect of an extra single shock were made. One observation was rejected because the interval following the extra beat was in this case four times the usual length. Over this series of fifty-nine experiments the mean interval following the extra beat was significantly longer than either of the two control intervals recorded before the extra beat (see Table 1, series 1). The difference between  $2.94 \pm 0.24$  sec.

Table 1. *The effect of extra beats on the rhythm*

Mean interval lengths, averaged over the number of repetitions shown, are given in lines *A*, *B* and *C* for the three normal intervals preceding the stimulus. The interval of line *D* is that curtailed by the stimulus, which initiates the number of extra beats shown. The mean intervals following are shown in *E*, *F*, *G* and *H*. The figures at the foot of the table which show a statistically significant change are in black.

Series Species	1 <i>Cyanea</i>	2 <i>Aurelia</i>	3 <i>Aurelia</i>	4 <i>Cyanea</i>	5 <i>Cyanea</i>	6 <i>Cyanea</i>
Repetitions of the sequence	59	20	10	8	10	10
Sequence of preceding mean intervals:						
<i>A</i>	$3.0 \pm 0.21$	$3.92 \pm 0.84$	$4.05 \pm 0.85$	$3.0 \pm 0.25$	$19.3 \pm 9.1$	—
<i>B</i>	$2.96 \pm 0.23$	$3.85 \pm 0.63$	$3.8 \pm 1.18$	$3.13 \pm 0.20$	$19.4 \pm 11.2$	$19.5 \pm 9.0$
<i>C</i>	$2.94 \pm 0.24$	$3.85 \pm 0.8$	$4.3 \pm 0.93$	$2.93 \pm 0.16$	$22.2 \pm 8.0$	$20.2 \pm 10.3$
Mean curtailed interval: <i>D</i>	$1.55 \pm 0.25$	$1.85 \pm 0.8$	2 sec. intervals	2 sec. intervals	2 sec. intervals	2 sec. intervals
No. of extra beats	1	1	8	3	10	20
Sequence of the following mean intervals:						
<i>E</i>	$3.68 \pm 0.43$	$5.22 \pm 1.32$	$6.8 \pm 6.3$	$14.25 \pm 8.1$	$31.5 \pm 16.8$	$51.0 \pm 25.6$
<i>F</i>	$2.9 \pm 0.24$	$5.22 \pm 1.32$	$11.0 \pm 4.3$	$3.25 \pm 0.75$	$33.5 \pm 14.9$	$38.0 \pm 13.9$
<i>G</i>	$2.97 \pm 0.2$	$4.27 \pm 1.0$	$5.75 \pm 1.7$	$5.88 \pm 5.1$	$37.5 \pm 13.2$	$20.2 \pm 11.1$
<i>H</i>	—	—	$4.45 \pm 1.25$	$3.13 \pm 0.20$	$18.5 \pm 8.2$	$17.7 \pm 6.5$

and  $3.68 \pm 0.43$  sec. for  $N = 59$  is significant to better than 99.9 %. As for the pause being compensatory,  $3.68 + 1.55$  cannot be shown to be different from  $2.94 + 2.96$  on the above evidence. Here lies the fallacy of trying to demonstrate a compensatory pause. The data, by their very nature, cannot show that the pause compensates for the previous shorter interval because this is the null hypothesis, i.e. in instances of apparent compensation the only statement that can be made is that there is no significant difference between the sum of the means  $B + C$  and the sum  $D + E$  (the letters refer to those of Table 1). Compensation can never be demonstrated. Some examples of Scyphozoa ganglia give a significant difference; i.e.  $B + C$  may be greater or less than  $D + E$ . Similarly, it cannot be shown that the rhythm is

reset at *the same* frequency; i.e. that  $E$  is equal to  $A$ ,  $B$  and  $C$ , for this again is a null hypothesis; certainly in some ganglia  $E$  cannot be shown to be different from  $A$ ,  $B$  or  $C$ , particularly when the rhythm is rapid, but it cannot be shown to be the same.

The example of *Cyanea* rhythm set out as series 1, Table 1, shows a slowing of the rhythm for one interval. A record which shows that the rhythm may be slowed for longer periods is set out as series 2 of Table 1. While the experiment was in progress the rhythm appeared to slow after each stimulus. A later test for significance showed that  $E$  is not greater than the mean intervals before the extra stimulus.

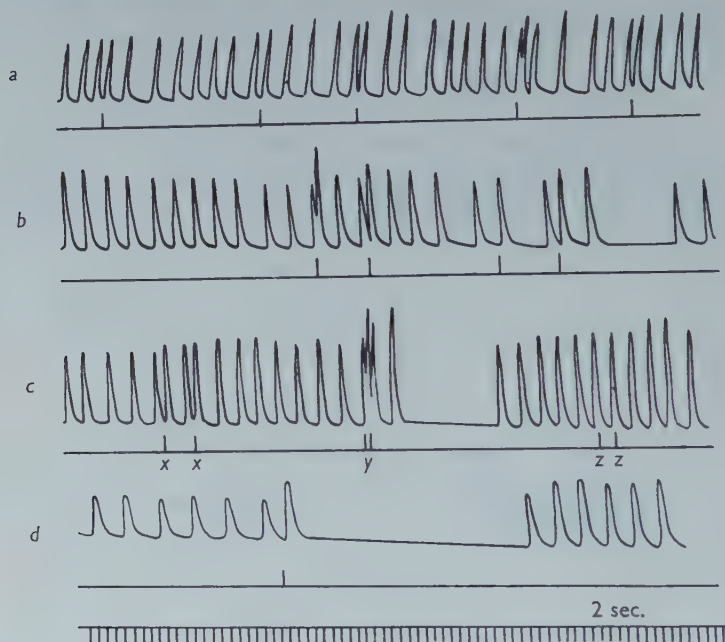


Fig. 4. The effects of an antidromic impulse on the rhythm of *Cassiopea*. This is a tracing from a kymograph record; for further explanation see text.

However, the sum of lines  $E$  and  $F$  is significantly greater than the sum of lines  $A$  and  $B$  (99 % level of significance). Therefore the slowing of the rhythm can be shown over the aggregate period of two intervals. The increase in standard deviation for lines  $E$  and  $F$  is also significant (99 % level). Therefore the rhythm becomes more variable. Twenty repetitions of this experiment just give a sufficient level of significance in face of the variability of the rhythm.

From *Cassiopea andromeda* I have available the results of about fifty experiments in the form of smoked-drum records, four of which are shown in Fig. 4. The segment of the *Cassiopea* bell was attached to a kymograph lever by the jelly, but nevertheless the rhythm was usually rapid and regular.

An extra beat introduced into the rhythm is followed by a variety of possible effects. Fig. 4(a) shows four instances in which the interval following an extra beat

was apparently normal and one instance in which it was shorter. In (b) the first two extra beats were followed by apparently normal intervals; the third and fourth were followed by a slowing of the rhythm. In (c) the results show the inconsistency of experiments on the rhythm. At X two single extra beats were injected and were followed by apparently normal intervals. At Y two extra beats were injected together. The following interval is normal but the next is longer. At Z two shocks of the same strength as before were given while the muscle was in the refractory period of a spontaneous beat. There was no effect on the rhythm though the extra height of the following contractions shows that the primary net was stimulated. In (d) a single extra beat was followed by a long pause and the regular rhythm suddenly started again at a slightly higher frequency than before.

The idea of a compensatory pause was based on experiments with *Rhizostoma*, and Bethe found that the pause was by no means always exactly compensatory. Pantin & Vianna Dias (1952) worked with *Aurelia* in Brazil; Horstmann (1934), who also found no evidence of a compensatory pause, worked on *Aurelia* of the North Sea. These results are the only primary data available. Bozler (private communication) tells me that he made no kymograph records of medusae. Ross (private communication) found the typical result for rapidly beating *Rhizostoma* to be a reset of the rhythm. We must conclude therefore that an apparently compensatory pause cannot be found, although an unexplained pause in the rhythm is consistently observed in most specimens.

#### (c) Several antidromic impulses

A number of beats may be artificially introduced into the rhythm, thereby forcing a higher frequency of discharge by an external pacemaker. The intervals following the extra beats are usually longer than normal; the effect is more marked than in experiments with one extra beat, i.e. fewer repetitions are required for a given level of significance and the rhythm continues for more than one interval at a frequency lower than normal.

Typical results are shown for *Aurelia* and *Cyanea* in Table 1. Following eight forced beats at 2 per sec. (series 3) *Aurelia* showed a slowing of the rhythm for three intervals following the final forced beat. The example of *Cyanea* shown in series 4 was beating rapidly before each experiment; the examples 5 and 6 were beating very slowly but the results do not appear to differ. There is a similar significant temporary slowing and increase in variability.

#### DIRECT CURRENT STIMULATION

Between each pair of radial muscles of *Cyanea* lies a muscle-free area in which there are many large bipolar cells running predominantly from the ganglion towards the muscles. This anatomical arrangement makes it possible to stimulate muscle plus nerve nets, nerve nets alone or ganglion plus nerve nets. As would be expected, condenser shocks in the above situations produce a contraction wave. A shock applied to the primary net where it occurs alone, as in the marginal lappets, can initiate a beat only indirectly from the ganglion after a marked delay of up to 3 sec.

The above experiments have been repeated with direct-current stimulation, using non-polarizable  $\text{Zn}/\text{ZnSO}_4/\text{Agar}$  electrodes.

When the ganglion has been removed, d.c. stimulation of the area of the giant fibre net may produce a contraction wave when the current is switched on but repeated contractions do not follow. The result is the same if the current flows through an area which includes muscle fibres. If there is a repetitive response of the giant fibre net it must be completed before the muscle recovers from its refractory period of about 2.0 sec. With the ganglion intact, d.c. stimulation of any region outside the ganglion may influence the rhythm, exactly as described for other stimulation; presumably the stimulus acts on the primary net.



Fig. 5. *Cyanea*. The effect of d.c. stimulation in the region of the ganglion (a) subumbrellum (SU) positive; current of 0.08 mA./mm.<sup>2</sup>. (b) SU negative; current 0.08. (c) SU negative; current 1.2. (d) SU negative; current 2.2; (e) SU negative; current of 2.2 mA./mm.<sup>2</sup> now slows down a spontaneous rhythm.

With the current passing through the stalk region of the ganglion the rhythm is accelerated when the negative electrode is on the exumbrellar side. With the subumbrellar electrode negative a greater current density is required and results are less consistent: an existing spontaneous rhythm may be stopped and repetitive discharges can also be evoked. Three effects on the ganglion are shown in Fig. 5 as demonstrated in one preparation. The current density required to show these effects is of the order of 0.1 mA./mm.<sup>2</sup>. The distribution of current between the inside of the nerve cells and the outside is unknown, and it is therefore impossible to assess conditions under which the nerves are stimulated.

An attempt to explain these results is partly frustrated by the action of the primary nerve net. The directional effect indicates an orientation of the pacemaker, but could equally well arise from orientated sensory cells in and around the ganglion. When the ganglion itself is stimulated by d.c. the effects persist for many beats; stimulation of the ganglion via the extraganglionic primary nerve net has only a transitory effect. However, the repetitive discharge of the stimulated ganglion may still originate indirectly in the ganglionic sensory cells which have shown themselves

to have a maintained effect on the rhythm in responses to light and gravity. At present I cannot show that d.c. stimulation of the ganglion has a primary effect on the pacemaker though the observations do not contradict this view.

## RESPONSES TO GRAVITY

### (a) *Compensatory movement*

When tilted from an even keel most Scyphozoa (not *Aurelia*) respond by adding an asymmetrical component to their normally symmetrical contractions. The uppermost edge relaxes more slowly and has a feeble stroke than the lower edge. Bozler (1926) found that the response depends on sensory cells in the region of the marginal ganglia; Horridge (1956*b*) inferred that the asymmetrical extra component of the contraction is transmitted via the primary (diffuse) nerve net and not by the giant fibre net. The compensatory response is not necessarily accompanied by changes in the frequency of the rhythm.

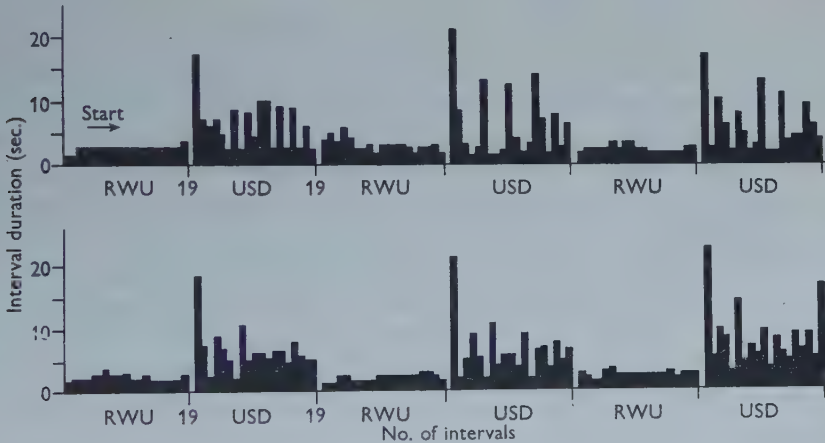


Fig. 6. The effect of inversion on the rhythm of *Aurelia*. The ganglion was alternately upside down (USD) and right way up (RWU) for 20 beats in each position. The durations of successive intervals are plotted upwards as columns of equal width reading from left to right.

### (b) *The effect of inversion on the rhythm*

The rhythm of a segment of *Aurelia* containing a single ganglion was recorded, and after every twenty beats the preparation was turned over so that the sub-umbrellar surface was alternately facing upwards and downwards. The frequent changes stimulate the rhythm so that the results cannot be compared with the normal experimental rhythm. Out of twenty preparations eleven consistently showed a slower and more variable rhythm when inverted. A typical record is plotted in Fig. 6. For at least the first twenty beats the rhythm was rapid and regular when the preparation was right way up; slower and irregular when inverted. However, when the same ganglion was allowed to remain right way up for longer, the rhythm progressively became slower and less regular. Starting to count after

30 min., the mean interval between beats was  $5.1 \pm 2.1$  sec. ( $N=100$ ) compared with a mean interval of  $6.9 \pm 2.9$  sec. ( $N=100$ ) of the inverted ganglion, similarly starting to count after 30 min. Such behaviour is not always found; of twenty ganglia from three *Aurelia* only eleven clearly showed a difference similar to that shown in Fig. 6. However, these preparations each behaved consistently, and the change of frequency must be considered as part of the possible behaviour of the ganglion.

The rhythm of an intact animal is determined by its fastest ganglion and therefore the acceleration of a single ganglion on turning right way up could accelerate the rhythm and hence the movement through the water. As yet there is no evidence that *Aurelia* has a steering mechanism; however, if it were turned haphazardly in all directions by water currents or by irregularities of the bell it would nevertheless tend to swim upwards. The above observations on the rhythm of a single ganglion suggest that *Aurelia* maintains its position in the sea by a *kinesis* in the sense defined by Fränkel & Gunn (1940).

#### THE ACTION OF LIGHT

Horstmann (1934*a*) describes the action of light on the rhythm of *Aurelia*. When daylight was allowed to enter a darkened room the frequency increased by a factor of 1.1–2.0. The number of beats per minute was recorded for up to 30 min. In eleven out of fifteen ganglia this effect of light was definite. I can now confirm Horstmann's observation in that several single ganglion preparations of *Aurelia* slowed their rhythm when shaded from daylight, for example a mean interval  $5.1 \pm 1.85$  sec. in daylight slowed to  $7.2 \pm 1.92$  sec. in the dark, measured for 40 beats.

Variation of light intensity appears to have no action on the ganglia of *Cyanea*, which differ from those of *Aurelia* in having no pigment spots (Horstmann, 1934*a*). The effect of an increased light intensity on *Aurelia* is an acceleration of a symmetrical beat, and the response of the intact animal is apparently a change in rate, not in direction of swimming.

The response to light of the ephyra larva of *Cassiopea andromeda* has a very marked directional character. When the subumbrellar surface is illuminated the animal turns through  $180^\circ$  in as few as four beats. The asymmetry of the contraction at each beat is similar to that found in the compensatory response to tilting of the axis (e.g. *Cyanea*, Horridge, 1956*b*) and the local component of the asymmetrical movement must presumably be controlled via the double innervation of the bell muscles.

#### THE ACTION OF TRYPTAMINE AND OTHER DRUGS

Following Ross's (1957) discovery that tryptamine is physiologically active on anemone neuromuscular preparations I tried its effect on the scyphozoan ganglion. The procedure was designed to take account of the following conditions. The rhythm must be observed for several minutes before a significant effect can be detected, but the control ganglion does not necessarily keep a constant rate for long periods; measurement of the duration of every interval is an unnecessary labour

when only the effect on the average rate is sought, but some measure of the variability is required.

A segment (*A*) of *Cyanea* bearing a single ganglion was placed in a large dish of sea water as control. Another segment (*B*) of the same animal with a rhythm similar to the first was placed in a similar dish which contained tryptamine diluted in sea water. The number of beats per minute was recorded for each preparation. In the

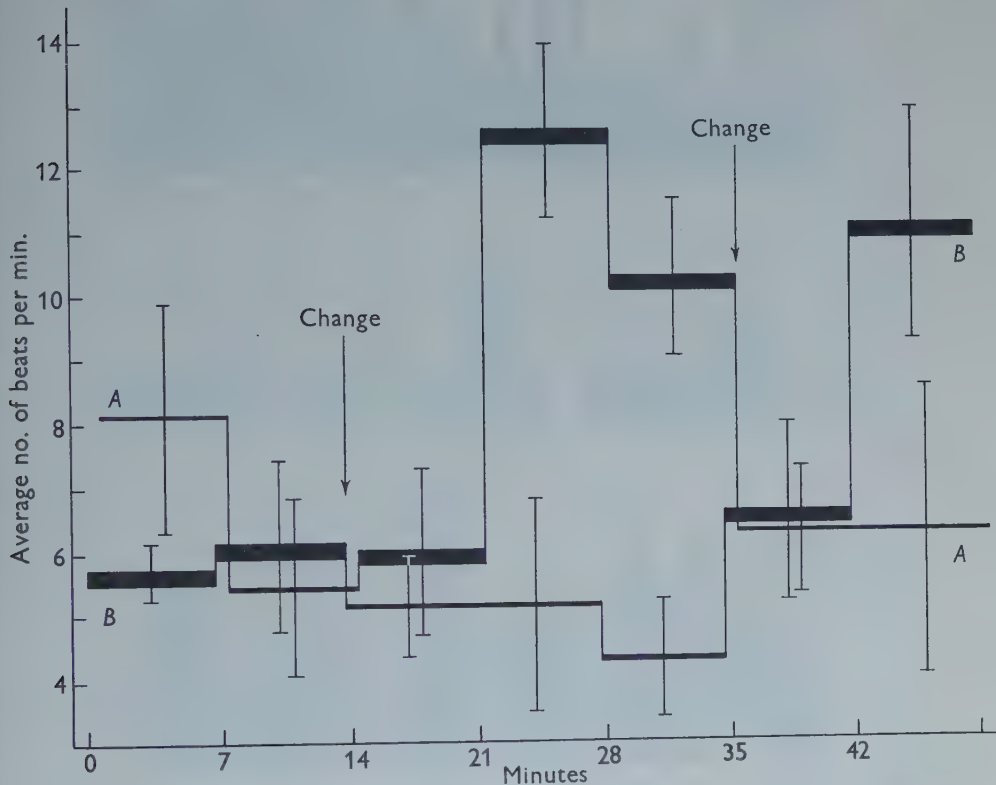


Fig. 7. The effect of tryptamine in concentration  $3 \times 10^{-6}$  g./ml. on the rhythm of two segments of *Cyanea*, *A* and *B*. The number of beats each minute was counted. The mean for each period of 7 min. is represented by a horizontal line; the standard deviation between seven observations each of 1 min. is given for each 7 min. period. A thick line indicates that the preparation is in the tryptamine solution, a thin line indicates that it is in sea water.

example set out in Fig. 7 this was continued for 14 min. The segments were then changed over, each into the other dish, and the rate per minute was recorded for 21 min. The segments were then changed back and observed for another 14 min. The drug acts slowly and there may be contamination between the dishes but both these objections can only reduce the effect of the drug as compared with the control. For convenience the results have been arranged (Fig. 7) as the average number of beats per minute for each 7 min. period, with standard deviation between minutes based on 7 min. in each case. The thick lines of the figure indicate that the ganglion

was temporarily immersed in the tryptamine solution. In each period except the first the treated ganglion beat faster, but the results become significant only when the treated ganglion has been immersed for at least 7 min.

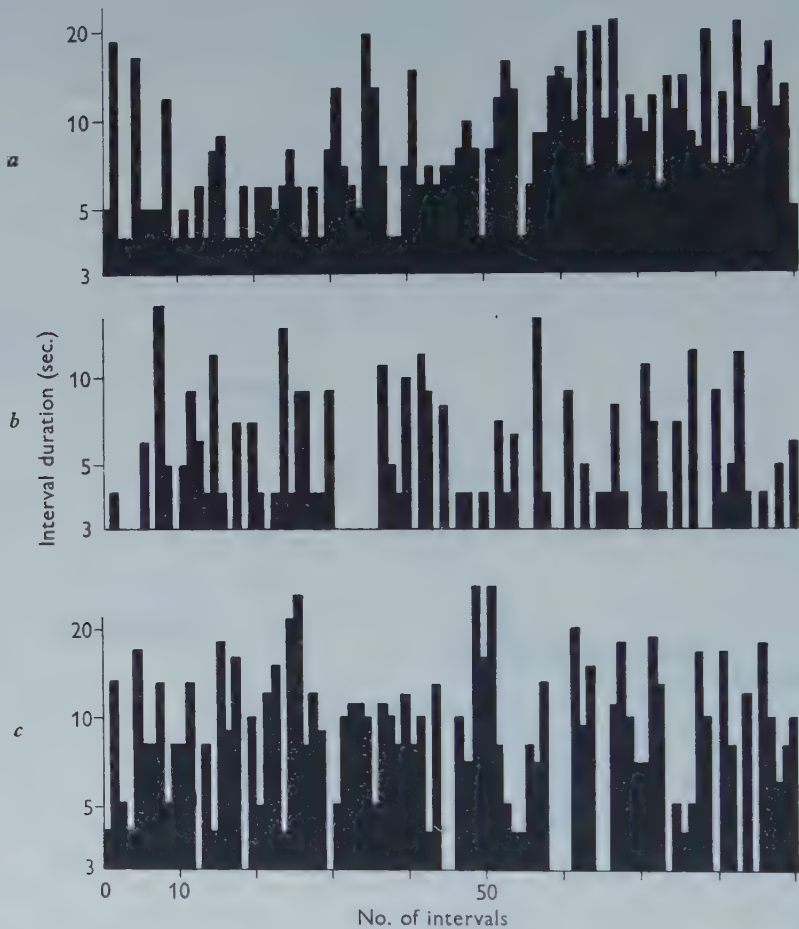


Fig. 8. The effect of tryptamine in concentration  $2.5 \times 10^{-5}$  g./ml. on the character of the rhythm of *Cyanea*. The durations of successive intervals are plotted upwards on a logarithmic scale as columns of equal width reading from left to right. The arbitrary base line was determined by the shortest interval, which happened to be 3 sec. (a) Initially in sea water, (b) after 12 min. in tryptamine  $10^{-4}$  g./ml., (c) on return to similar sea water.

Tryptamine accelerates the rhythm and has been effective on *Cyanea* in concentrations as low as  $10^{-5}$  g./ml. of sea water. *Aurelia* is 10–100 times less sensitive. The effect is completely reversible. No effect on the rhythm has been detected apart from the increased rate. A record of intervals for a typical example is shown in Fig. 8. In particular, analysis of three likely records failed to detect any effect on the first-order serial correlation coefficient. Apparently the drug acts directly on the pacemaker.

By use of the above method of interchanging the control and the treated ganglia between two dishes the effects of the following drugs at concentrations of  $10^{-3}$  g./ml. were tested but no significant influence on the average frequency was discovered: acetylcholine with and without physostigmine, adrenaline, curare, ephedrine, histamine, 5-hydroxy-tryptamine. These observations suggest that, like the coelenterate neuromuscular junction, the pacemaker is pharmacologically different from analogous mechanisms in other phyla.

#### THE RHYTHM OF AN INTACT ANIMAL AND OF A SINGLE GANGLION

Several attempts to demonstrate some degree of co-ordination between the rhythms of the eight to thirty-two ganglia round the margin of the bell have been reported. Two mutually compatible views can be found in Romanes (1877). He suggests that the rhythm originates from afferent excitation which comes into each ganglion from all parts of the bell, and also that the fastest ganglion fires off all the others and superimposes its rate on them. Horstmann (1934*a, b*) comes to the conclusion that continuous sensory excitation is converted in the marginal ganglia to rhythmical motor excitation. He also notes that the rhythm of the whole animal is more regular than that of a single ganglion. To account for the latter fact he suggests that a variation of frequency of one ganglion is compensated by an appropriate opposite variation of other ganglia which he supposes to be co-ordinated with it. Another suggestion of Romanes is that the rhythm is co-ordinated by some property, such as refractory period, of the muscle sheet, a suggestion which could only apply at frequencies higher than those considered in this paper. Fränkel (1925) suggests that the regularity of the beat has its origin in the movement of the bell itself because the tentaculocysts swing in unison and so maintain exact synchrony.

The above statements must be revised in the light of more recent work on the two overlying nerve nets of Scyphozoa. Excitation in each of the two nets has a particular effect on the rhythm of a ganglion. The action of the giant fibre net in resetting the rhythm of all but the fastest ganglia is experimentally unavoidable, so that the potential rhythms of the slower ganglia are never observed unless the ganglia are isolated. Horstmann found that first one ganglion then another initiates a contraction wave and supposed that some mechanism other than the contraction wave itself was responsible. However, he ignored the standard deviation between intervals and the other characters of the rhythm now described. A further objection to any theory of co-ordination between ganglia is raised by the observation that in favourable specimens of *Aurelia* the excitation in each conducting system is accompanied by a visible wave of contraction, in one case of the bell muscle, in the other of the marginal tentacles; yet during normal swimming the tentacles are relaxed and extend passively to their full length. This indicates that the primary (diffuse) net does not participate in co-ordination of the ganglia. Lastly, Fränkel's suggested mechanical mechanism of co-ordination has not been tested by experiment; for example, the bell might be clamped in various ways to bring out the effect of different movements.

If the ganglia are separately observed in isolation, each will show a normal experimental rhythm. Fig. 9(a) shows a typical example of the distribution of the various intervals between beats for one typical ganglion. If eight such ganglia were co-ordinated by a pathway with the properties of the giant fibre net then the mean interval between beats would be shorter than that for the isolated ganglion. This is

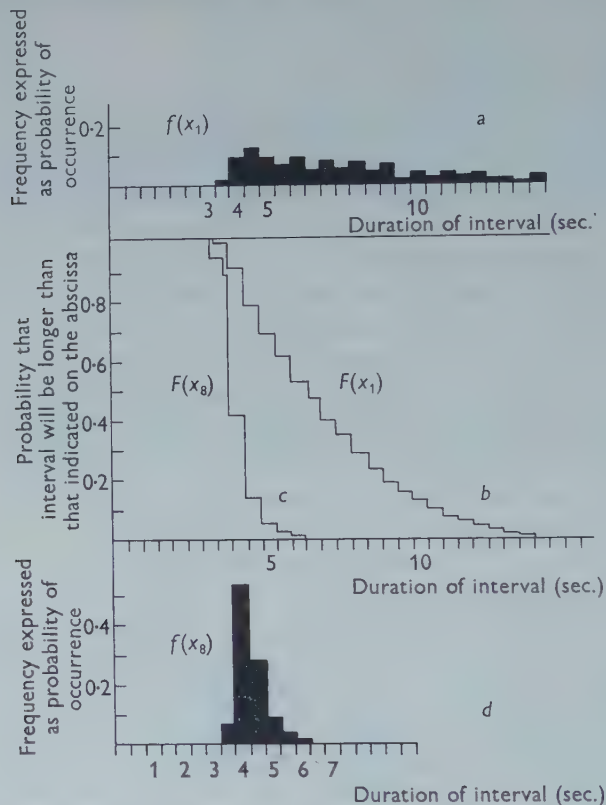


Fig. 9. Derivation of the theoretical distribution function (d) of intervals for a jellyfish of eight similar ganglia from the observed distribution (a) of intervals of one of the ganglia. For further explanation see text.

because the probability of any particular interval being short is increased by the presence of several ganglia, and the short interval determines the rate for all. The calculation of the distribution of intervals for eight connected ganglia is as follows:

The distribution of intervals for a single ganglion is drawn,  $f(x_1)$  (Fig. 9a). From this the cumulative distribution function  $F(x_1)$  is drawn out by summing the occurrences from  $P=0$  for the longest intervals to  $P=1$  for the shortest intervals. The ordinate is the probability that values will be greater than the readings on the abscissa (Fig. 9b). The probability that a particular interval will be greater than a particular value  $x$  will be  $F(x)$ . When  $n$  ganglia are considered the probability that *all* will have an interval longer than  $x$  will be  $(F(x))^n$ . This means that the long intervals become less likely, for  $(F(x))^n$  is less than  $F(x)$ : therefore short intervals are more

likely. In Fig. 9(c) is drawn  $F(x_8)$  for eight ganglia and from the differences between the heights of the steps of  $F(x_8)$  the distribution  $f(x_8)$  is drawn in Fig. 9(d).

Comparison of  $f(x_8)$  with  $f(x_1)$  shows that the rhythm of the eight combined ganglia is less variable than that of a single ganglion, although there is no separate excitation which co-ordinates the ganglia. In this example the mean observed interval is  $6.93 \pm 2.5$  sec. for a single ganglion and the mean calculated interval for eight ganglia is  $4.23 \pm 0.63$  sec. ( $N = 122$  in each case).

This theory is complicated by several factors. The negative serial correlation coefficient  $r_1$  means that short intervals will not occur as often as expected on the above theory because they will tend to be followed by long ones in all the connected ganglia. However, since  $r_1$  is small the effect has been disregarded. Secondly, the independent rhythms of the ganglia differ. This only means more labour in that  $F(x_1).F(x_2).F(x_3)$ . etc, is substituted for  $(F(x))^n$ .

#### THE REVERSAL OF THE COMPENSATORY MOVEMENT

Fränkel (1925) first showed that the beat of the jellyfish *Cotylorhiza* is asymmetrical when the animal is tilted from an even keel. This species always tended to swim upwards in his experiments. The *Rhizostoma* which he observed swam horizontally and failed to give a compensatory movement towards the vertical. However, he adds that in 'special physiological states' *Rhizostoma* shows a compensatory movement.

During observations of several species of Scyphozoa certain findings suggest something comparable to Fränkel's 'physiological states'. For example, specimens of *Pelagia noctiluca* at Naples in 1957 swam upwards when left undisturbed and downwards when held by a clip for better observation. Bozler (1926*b*) found that his specimens of *Pelagia* swam downwards when stimulated, though in the previous year they swam upwards. *Cassiopea andromeda* normally rests subumbrellar surface uppermost on the bottom in shallow water. When disturbed it swims in the normal way upwards and then turns over and swims to the bottom upside down. *Cyanea* specimens usually swim upwards in the laboratory but when disturbed they can be induced to turn over and swim downwards, and when left in water some individuals persistently hit the exumbrellar surface on the bottom of the tank. The mechanism of the reversal of the compensatory movement is unknown; presumably it resides in the marginal ganglia.

#### CONCLUSIONS

##### *The regulation of the rhythm*

The sequence of intervals is not haphazard, and one particular regularity is shown by the negative first-order serial correlation coefficient; long intervals tend to be followed by short ones and vice versa. A self-regulating action of the ganglion is also shown by the temporary slowing which follows a period of abnormally high frequency; a series of artificially initiated beats has a greater effect than a single extra beat. The same regulatory mechanism could account for both of the above effects and the pause which follows a series of forced beats is the overshoot of an

inadequately damped regulator. A similar overshoot is seen in the return to normal frequency in Fig. 3. If such a regulatory system becomes over-compensatory the result could be a regular cyclical series of bursts of beats; and in fact a cyclical rhythm is commonly observed in over-stimulated or damaged preparations. The rhythm is normally controlled at a frequency below the maximum.

### *Organization of the ganglion*

The marginal ganglion of the jellyfish is a concentration of nerve cells on the stalk of the tentaculocyst and in the neighbouring epithelium. It appears to be a relatively simple nervous centre whether considered in terms of histological structure or functional capabilities. The ganglion is connected to the rest of the animal by two physiological conducting systems which are both nerve nets (Horridge, 1956 *b*). A study of the marginal ganglia of the *Aurelia* ephyra revealed only four types of neurones (Horridge, 1956 *a*), and though sensory cells are more numerous in the ganglion of the adult there is no physiological indication that the organization is fundamentally different. The ganglion has two characteristics of higher central nervous systems; it produces impulses spontaneously and it integrates several kinds of sensory excitation, each of which alone can influence the rate of the spontaneous rhythm. The ganglion was considered by Jordan (1912) to be a rudimentary reflex centre, but this term is avoided here on account of its associations with the study of the vertebrate nervous system.

The data now presented show the responses of the ganglion to a variety of stimuli so far as an influence on the rhythm has been noted. The results do not conflict with the histological and physiological results previously set out for the ephyra larva; a physiological model of the ganglion can be partially correlated with a histological map of neurone connexions. Histologically, in both the adult and the ephyra ganglia, there are central endings of motor cells of the giant fibre net. The most satisfactory physiological model locates the pacemaker at these endings, with a polarized junction separating it from the sensory inputs. At this junction the sensory excitation from several distinct sources would be integrated together to modulate the rhythm. The physiological results can be interpreted in terms of one such integrating junction and one pacemaker in each ganglion. However, to account for the large number of nerve cells in the tentaculocyst, a reduplication of pacemakers in the ganglion is suggested, with the fastest setting the pace as for the interaction between ganglia.

The information available from the present study relates only to the rhythmical output of the intact ganglion. Such observations cannot demonstrate the internal organization but may be used to test a theory suggested on other grounds. Experimental confirmation of the mechanism must spring from observations on the components of the ganglion. The theory suggested above has no reference to the self-regulatory mechanism which is inferred from the observations of the sequence of intervals. Here we meet a difficulty in the analysis of the normal action of the ganglion. Experiments that separate the pacemaker from its regulator will produce an abnormal rhythm; but on the other hand the system as a whole defies analysis.

This is a property of any self-regulating mechanism; that this feature would appear in such an elementary central nervous system was unexpected.

### SUMMARY

1. Features of the rhythm of isolated ganglia of *Aurelia* are the high variability of the intervals between beats and their negative first-order serial correlation coefficient.
2. A regular rapid rhythm or a cyclical rhythm is a consequence of stimulation.
3. The effect of stimulation of the primary (diffuse) nerve net is usually a transient acceleration of the rhythm.
4. A rapid, uniform rhythm is usually reset at a similar frequency by an artificially induced beat; a normal rhythm usually shows a slight pause, which is accentuated if several antidromic impulses are initiated.
5. The pause which follows forced beats, the negative serial correlation coefficient and the time course of the return to normal after stimulation indicate a self-regulating mechanism within the ganglion.
6. Stimulation of the ganglion with direct current can stop or accelerate the rhythm but throws no light on the possible mechanism.
7. When the ganglion is inverted the rhythm becomes slower and more variable.
8. Tryptamine accelerates the rhythm. The minimum effective concentration in the bathing sea water was  $10^{-5}$  g./ml. Acetylcholine, adrenaline, curare, ephedrine, histamine, 5-hydroxy-tryptamine and physostigmine have no effect.
9. The theoretical relation between the rhythm of one ganglion and of eight connected ganglia shows that the apparent redundancy of ganglia gives a more regular rhythm.

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# RESPIRATION RATES OF WORKER HONEYBEES OF DIFFERENT AGES AND AT DIFFERENT TEMPERATURES

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## INTRODUCTION

Since Rösch (1925) published his results on the ages of the worker honeybees (*Apis mellifera* L.) performing various duties in the colony, considerable interest has been focused on this problem. While it now seems clear that there is no rigid demarcation between the ages of adult workers engaged on different activities—for one bee may perform several different duties on the same day (Lindauer, 1952)—it nevertheless appears that age is not without influence in this respect, as for example in the feeding of the brood (Perepelova, 1928), the feeding of the queen (Allen, 1955), foraging (Ribbands, 1952) and 'shaking' (Allen, 1958). In view of the present state of some uncertainty on the extent of this influence it seemed of interest to examine the metabolic rates of workers of different ages, as reflected by their respiration rates, with the aim of discovering whether any marked differences existed which might in turn be related to the problems of behaviour.

With many animals, especially mammals, it is apparently true that in general the respiration rate falls as the animal becomes older and also as it grows larger, though this is not by means always the case, as for example in a number of invertebrates (reviews by Heilbrunn, 1952, Ch. 20; Prosser *et al.* 1950, Ch. 8). Also, in probably the majority of invertebrates and poikilothermic vertebrates the respiration rate increases with temperature, within the vital limits, although the value of the temperature coefficient does not necessarily remain constant at different temperatures. Honeybees and some other winged insects have, however, the ability to raise their temperature appreciably above that of the environment (Pirsch, 1923; Himmer, 1932) and their metabolic rate is, therefore, likely to bear a complex relationship to environmental temperature. Bearing this in mind it seemed of some interest to relate the oxygen consumptions of stationary worker bees not only to age, but also to external temperature over the range between the point at which the insects were just in chill coma and the estimated upper lethal temperature (approximately 7–46° C.).

## METHODS

Newly emerged bees were marked with spots of distinctively coloured paint on the thorax, and left in their parent hives until required for the experiments. For the determinations of respiration rates the bees were captured and each was placed in

a separate match-box and fed with honey for 10 min., after which it was picked up by the wings and was put head-first into a copper gauze cylinder approximately  $\frac{1}{4}$  in. in diameter and 1 in. long, which had one end sealed off. With blunt forceps the bee was gently pushed down the cylinder until its head was just touching the end, and then a plug of cotton wool was inserted so that it rested lightly against the tip of the abdomen and closed the cylinder. As a result only slight movements were possible. Throughout the caging operation there was a danger that the bee might use its sting and in so doing be damaged by having it dragged out of the body, but with practice this did not occur frequently. However, when any bee was damaged in this way (or in any other) it was discarded.

It was appreciated that by feeding the bees before the experiments the respiration rates would probably be raised somewhat above those of fasting bees, but since some bees (particularly incoming foragers) would undoubtedly have had some honey or nectar in their honey stomachs when they were removed from the hive the only means of achieving any uniformity in this respect seemed to be by supplying food to all bees prior to the measurement of their respiration rates.

It was in most instances found convenient to measure the respiration rates of two age-groups simultaneously, using six (or in a few instances, five) bees of each age; immediately after the bees were caged each one was placed in a separate Warburg constant volume respirometer with a capacity of between 24 and 29 ml., containing 0.2 ml. 5% KOH solution in the centre well, and the apparatus was then allowed to equilibrate at the experimental temperature for 15 min. before readings were commenced. The preparation time, by which is meant the duration of the period between the removal of the bees from the hive and the end of equilibration, was in all cases approximately 1 hr. All the experiments were carried out between 5 April and 12 September, when the colonies were in the active summer condition.

The respirometers were not shaken during the experiments because normal gaseous diffusion was sufficient to ensure a constant supply of oxygen to the bees. The volumes both of the bee (approximately 100  $\mu$ l.) and of the copper and cotton wool of the cage (approximately 100–160  $\mu$ l.) were not taken into account in the calculations of the flask constants, in view of the fact that they were relatively constant and in any case would have caused a reduction of less than 1% in the final result, although their accurate determination would have added considerably to the labour involved.

The oxygen consumptions were calculated on a unit weight basis, and it was found during the present experiments that worker bees varied in weight between 80 and 179 mg. This variability was due partly to inherent variation, partly to differences in age (Haydak, 1934), but very largely to differences in the volume of the contents of the honey sac and rectum. This latter factor, therefore, would have led to major errors in the calculation of respiration rates if the total weight has been used. In consequence the alimentary canals were removed before weighing the bees at the end of the experiments. The technique adopted was to kill the bee in ethyl acetate vapour, to cut off the head, and finally to pull out the complete alimentary canal with last abdominal segment, as described by Haydak (1934). The head,

thorax and abdomen (minus the last abdominal segment) were then weighed. It was not practicable to determine the weights of the cleaned alimentary canals of each bee used in the experiments, but an independent investigation showed that for a bee weighing 70 mg. (approximately the mean weight) after removal of the alimentary canal, its average weight was 9 mg. Accordingly, an addition of 13% was made to the weights of all the bees used.

After a number of preliminary experiments it was decided that there was a possibility of oxygen shortage influencing the results after about one-eighth of the total volume of oxygen had been consumed. In all, seventy-nine groups of bees (comprising 463 individuals) were used, and in fifty-four of these groups this point was not reached during the first hour. Here the final readings were taken after 60 min., but in some experiments the respiration rates were more rapid and then the final readings were normally taken after 30 min. Because of this and for other reasons twenty-three groups of bees were recorded over this shorter period, and two groups over 20 min. only (Table 1).

It is evident that if the individual respiration rates were in the process of increasing or decreasing in those experiments where respiration rates were measured over less than 1 hr., the calculated volume of oxygen consumed in one hour would be respectively either lower or higher than the volume which would have been consumed had it been possible to continue the experiments for the full hour: nevertheless, after an examination of the individual respiration rate curves it was considered preferable to use the values based on the greater numbers of readings rather than be limited to the results obtained after 30 min. in all cases, both because of the greater accuracy of the final result (particularly at low rates of respiration) and also in view of the fact that the causes of the increasing and decreasing respiration rates were not sufficiently well known.

## RESULTS

Using groups of bees ranging from 0 days old to 22–33 days old the relationships between the ages of the bees and their oxygen consumption per mg. live weight per hour ( $Q_{O_2}$ ) were determined at 7, 12, 17, 22, 27, 32, 37, 40, 42 and 46°C. Figs. 1 and 2 are given as examples of the results obtained.

The values of  $Q_{O_2}$  (see above) of individual bees at each particular age and temperature usually had a markedly skew distribution, that is, far more readings fell on the lower side of the mean than on the higher. In order to work with data having more nearly a normal distribution it was found convenient to use a logarithmic transformation when comparing age and temperature effects, and in Figs. 1 and 2 it is the logarithms (to base 10) of the individual  $Q_{O_2}$  values and the means of these which are shown. The fact which emerges clearly is that in spite of the variability of the individuals in each group there is a rise in the rate as between very young and older bees at each temperature. At all temperatures studied this rise invariably occurred during the first week of adult life, and there was apparently a broad tendency for it to continue throughout the next 3 weeks (after which experiments were terminated).

Table 1. *Details of ages of bees used in the experiments*

(Figures in brackets indicate replication of groups.)

Temperature (° C.)	Length of time over which respiration rates were determined		
	60 min.	30 min.	20 min.
7	Newly emerged bees (× 2) 18 hr. old bees 2-day-old bees 12-day-old bees 19-day-old bees 33-day-old bees	—	—
12	Newly emerged bees 18 hr. old bees 2-day-old bees 6-day-old bees 15-day-old bees 20-day-old bees 32-day-old bees	—	—
17	Newly emerged bees 18 hr. old bees 2-day-old bees	9-day-old bees 17-day-old bees	21-day-old bees 27-day-old bees
22	Newly emerged bees 18 hr. old bees 7-day-old bees	2-day-old bees 18-day-old bees 32-day-old bees	—
27	Newly emerged bees (× 4) 18 hr. old bees (× 2) 2-day-old bees (× 2) 4-day-old bees 6-day-old bees 10-day-old bees (× 2) 17-day-old bees 19-day-old bees 26-day-old bees 33-day-old bees	—	—
32	Newly emerged bees 7-day-old bees 14-day-old bees 20-day-old bees 23-day-old bees 30-day-old bees	18 hr. old bees 2-day-old bees	—
37	Newly emerged bees (× 3) 5-day-old bees 8-day-old bees 13-day-old bees	18 hr. old bees 2-day-old bees 22-day-old bees 29-day-old bees	—
40	Newly emerged bees 8-day-old bees 15-day-old bees 22-day-old bees	—	—
42	Newly emerged bees 17-day-old bees	18 hr. old bees 2-day-old bees 4-day-old bees 8-day-old bees 10-day-old bees 31-day-old bees	—
46	—	Newly emerged bees 18 hr. old bees 2-day-old bees 13-day-old bees 20-day-old bees 32-day-old bees	—

The chief interest of the respiration rates given here lies not so much in their actual numerical values as in the relative differences at different ages and temperatures, for it is not claimed that they are a measure of basal metabolic rates of the bees concerned but simply that they reflect the metabolic rates under the experimental conditions. Consequently, the results have been replotted in Fig. 3 to show

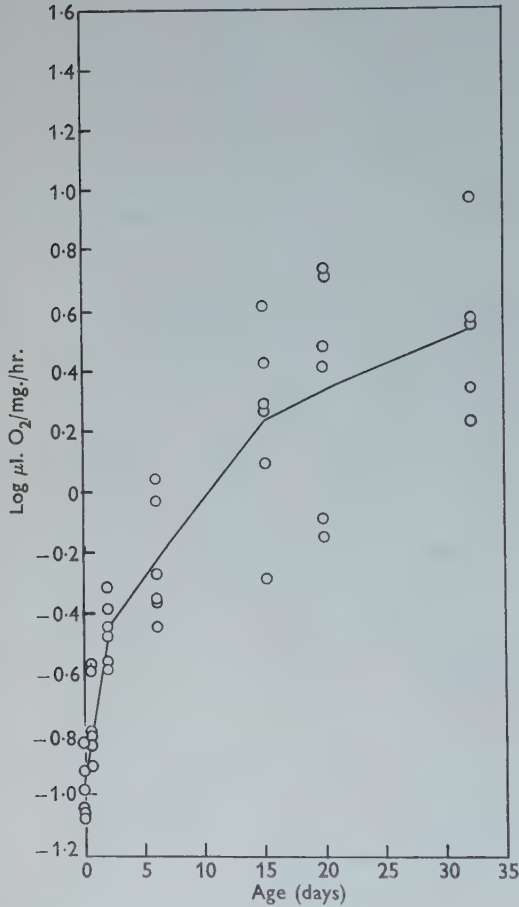


Fig. 1. Oxygen uptake of adult workers at 12° C.

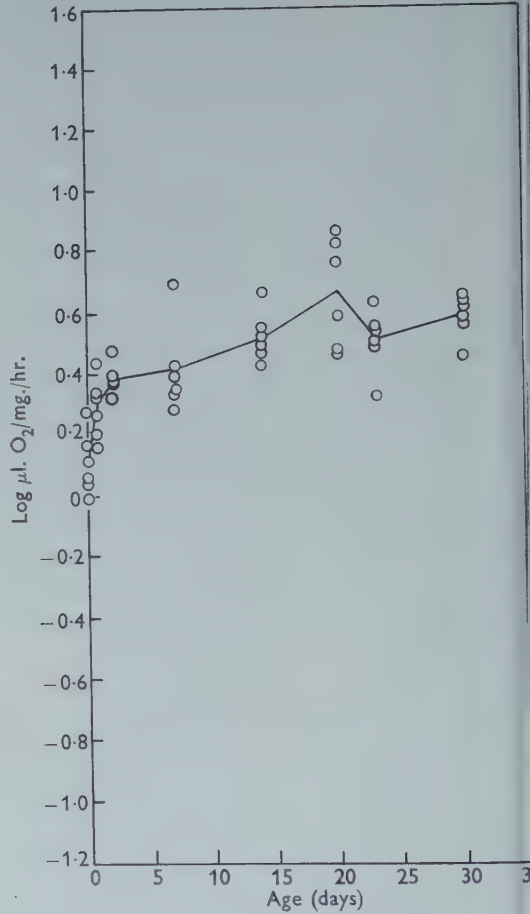


Fig. 2. Oxygen uptake of adult workers at 32° C.

the influence of both temperature and age on the respiration rates. The age groups here considered are as follows: newly emerged, 18 hr., 2 days, 4-13 days, 14-23 days, and 24-33 days. The means used (as in Figs. 1 and 2) were calculated from the  $\log_{10}$  respiratory values of all the individuals falling into each of the above categories.

The first point to be noticed in Fig. 3 is that, as already noted, the curves for the most part succeed each other in height as they do in age, the newly emerged bees occupying the lowest position and the 24-33-day bees the highest.

Secondly, the individual curves do not all follow identical paths. The newly emerged and 18 hr. bees have basically a linear increase in respiration rate with temperature when plotted in this way (apart from a few fluctuations), as might be expected in a poikilothermic animal. At 2 days old, however, the position is less straightforward, since there is a peak at 22° C. in addition to the peak at 46° C. This earlier peak is even more pronounced in the three oldest groups, where it occurs at 17° C., reaching a height approximately the same as that at 46° C. Between the two peaks is a trough with a minimum value at about 32° C.

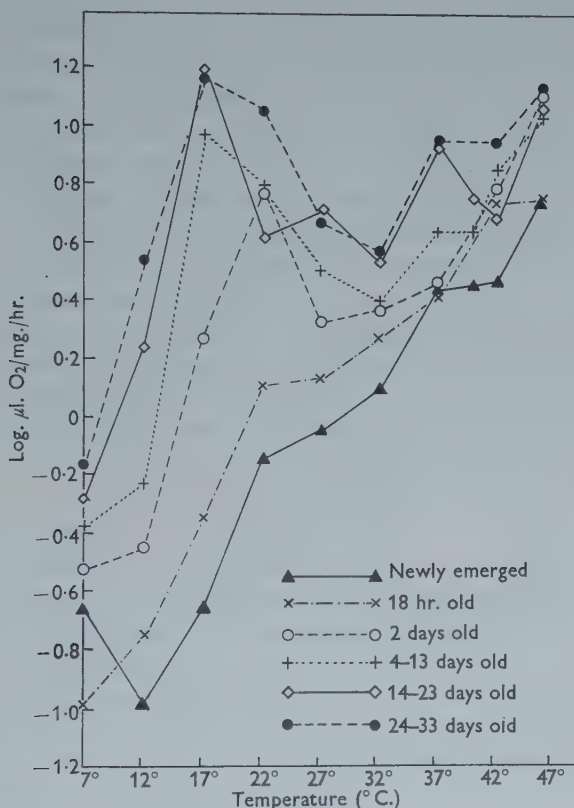


Fig. 3. Effect of age and temperature on oxygen uptake of adult workers (means).

The main trends shown in Fig. 3 are very clear, but it is perhaps informative also to consider the individual results briefly. Usually the readings for each bee recorded at 10 min. intervals lay closely on a straight line and sudden changes in respiration rate, which would have indicated fluctuations in activity, occurred only in six instances; of these, four were found at a temperature of 46° C., which was lethal after about 1 hr. Sometimes, however, a tendency for respiration rates either to increase or decrease with time was apparent; the details of these changes have been discussed by Allen (1958), but broadly speaking they amounted to a decrease with time at the lowest temperatures (7 and 12° C.; possibly at 17° C.), and an increase

at temperatures approaching the upper lethal point ( $42$  and  $46^{\circ}\text{C.}$ ), although not all bees at these temperatures showed any alteration in rate over the period studied. At  $22$ ,  $27$ ,  $32$ ,  $37$  and  $40^{\circ}\text{C.}$  there was no consistent indication of a curved relationship with time.

It has been stated already that there was little evidence of fluctuating muscular activity during the experiments, but no visual estimate could be made of the level of activity while the respiration rates were being measured because the bees were not clearly visible while in the gauze cages. However, in order to make some assessment of the probable activity, observations were made on groups of ten foraging and ten newly emerged bees confined in glass tubes by pads of cotton wool, so placed that the bees could make only limited movements. These were maintained at temperatures of  $7$ ,  $12$ ,  $17$ ,  $27$ ,  $37$  and  $46^{\circ}\text{C.}$  respectively. Both age groups were completely motionless at  $7^{\circ}\text{C.}$  but the foraging bees made slight movements at  $12^{\circ}\text{C.}$ , whereas the young bees were in a state of chill coma as before. At  $17^{\circ}\text{C.}$  the foragers were apparently capable of making normal movements, but the young bees reached the corresponding state only at  $22^{\circ}\text{C.}$  At  $27$  and  $37^{\circ}\text{C.}$  both groups moved readily if at all disturbed, but if left undisturbed both tended to remain stationary. At  $46^{\circ}\text{C.}$  both for a time showed marked respiratory movements of the abdomen and made rather spasmodic movements of the legs, though later the level of activity gradually decreased, until after 60 min. one newly emerged bee and six foragers were found to be dead. It thus appears that the foragers may be more susceptible than the young bees to high temperatures, whereas the young bees chill at low temperatures more readily than the old bees.

#### DISCUSSION

It has been shown that the respiration rates of the two youngest groups of bees studied (0–18 hr. old) had a simple relationship with temperature. All the groups of older bees, however, had two peaks, one in the region of the upper lethal temperature and the other at a clearly suboptimal temperature. The lowest point of the trough lay between at about  $32^{\circ}\text{C.}$ , which is the temperature found towards the centre of the brood area in a healthy colony. It is difficult or impossible to define the optimum temperature for adult bees, but at  $32^{\circ}\text{C.}$  the workers carry out many of their normal activities under natural conditions, and for this reason it may be assumed that at this temperature the respiration rates are those corresponding to the rates occurring in the brood area of the hive. For convenience, temperatures in the region of  $32^{\circ}\text{C.}$  will be referred to subsequently as 'optimal temperatures'. From this assumption and from the evidence supplied by the two youngest groups in Fig. 3, it would seem that the first of the two peaks in the older age groups can be regarded as an elevation of the 'expected' rate, due to factors not normally operating in truly poikilothermic animals. It is suggested that the cause is, in fact, that the bees are exerting some degree of temperature regulation by which the metabolic processes are speeded up when the environmental temperature is too low and the insects are consequently in danger of being rendered inactive.

The temperature at which confined bees actually chilled was shown to be notice-

ably higher in the newly emerged bees than in the foragers, and it is noteworthy that in the three oldest groups studied (4–33 days) the first peak is found at a temperature thought to be the lowest at which normal movements are possible. Furthermore, the 2-day-old bees, which are likely to chill at a temperature intermediate between that causing chilling in the youngest and in the older bees, showed this first peak at 22° C. instead of 17° C., presumably again indicating that it occurred as soon as the temperature was high enough to permit normal movement. The results also suggest that the movements (if, indeed, the increased respiration were due to greater muscular activity) decreased in vigour when the optimal temperatures were reached, since the oxygen consumption at these temperatures was lower than previously; whereas if the same degree of activity had been maintained throughout, oxygen consumption should have risen with increasing temperature.

It has been found previously by a number of workers that individual bees, as opposed to a group, can raise their internal temperature above that of the environment when it is at suboptimal levels and lower it at high temperatures as, for example, Pirsch (1923) has shown. In addition, Himmer (1932) reported that this temperature regulation in the honeybee was influenced by age. He demonstrated that 1–2-day-old bees had only a very limited ability to raise their temperature above that of the environment, while ‘house’ bees of 3–15 days were much better developed in this respect, and guard bees and foragers of more than 20 days old could raise their temperature even higher above that of the surrounding air. The results shown in Fig. 3 are in accordance with these findings, since the highest values for oxygen consumption at suboptimal temperatures were recorded in the two oldest groups of bees (14–33 days), with a steadily declining rate as age decreased. Also in agreement with Himmer’s work are the lack of evidence of temperature regulation in the two youngest groups (0–18 hr.), the peak at 22° C. in the 2-day-old group, and the earlier, higher peaks in the 4–33 day groups at 17° C.; all of which very probably indicate higher internal temperatures with increasing age at these suboptimal environmental temperatures.

Expressed in another way, this early peak and subsequent depression in the respiration rates of what would be the majority of the bees in a colony is almost certainly related to the heat regulation of the cluster. At temperatures below the optimal cluster temperature some means of heat production is necessary and, whatever the exact mechanism, this can be achieved in practical terms only by increased consumption of oxygen. At or about the optimal temperature, however, no such increased rate of consumption is required. The quieter behaviour of the bees observed in the glass tubes when at 27 and 37° C. than when at 17° C. is also broadly in keeping with this view.

It is difficult to compare the numerical values for oxygen consumption obtained in these experiments with those found by the few other workers who have made similar measurements on bees at various temperatures (Parhon, 1909; Steidle, quoted by Zander, 1921; Kosmin, Alpatov and Resnitschenko, 1932) because they have all used different methods of calculating the final  $Q_{O_2}$  values, and this materially affects the results. Furthermore, Parhon used relatively large groups of bees free

to move about, whereas Kosmin *et al.* (1932) worked with individual bees and noted the amount of movement. Steidle also used individual bees but did not disclose their degree of activity. The results of these workers have been further discussed elsewhere (Allen, 1958), but here it is sufficient to mention that there is some indication that the older bees in the present experiments had somewhat similar respiration rates to the foraging bees of Kosmin *et al.* (1932) which were described as 'moving slowly on floor of vessel'.

Finally, returning to the question of whether behavioural changes at different ages coincide with changes in metabolic rates, it can be stated that there is some evidence that this is actually the case. For example, the present results suggest that the older bees are capable of raising their internal temperature above that of the environment when the latter is at a markedly suboptimal level, whereas the younger bees show no sign of having such an ability, and it thus appears that the responsibility for raising the temperature of the colony in cold weather must lie with the older bees. Similarly, an appreciable difference has been demonstrated between the rates of oxygen uptake of young bees and that of bees of foraging age, and while this could possibly have been due to a greater degree of movement by the older bees, the fact that the same difference was found at 7° C. when all ages were presumably quite stationary would indicate that more basic causes were responsible.

#### SUMMARY

1. The respiration rates of individual adult worker bees confined in small gauze cages were determined over a range of temperatures between 7 and 46° C. The respiration rates usually remained constant over a period of 1 hr., although at 7, 12° C., and possibly at 17° C. there were indications of a decrease with time, and at 42 and 46° C. indications of an increase with time. Fluctuations which would have reflected variable activity during any of the experiments were rare.

2. At each temperature studied the respiration rates increased progressively from the youngest adults (newly emerged) to the oldest measured (24–33 days). The increase was particularly marked during the first few days of adult life.

3. The respiration rates of newly emerged and 18 hr. old adults increased progressively between 7 and 46° C., but 4–33-day-old bees showed two peaks (at 17 and 46° C.) with a depression having a minimum value at 32° C. lying between. 2-day-old bees had similar peaks at 22 and 46° C.

4. It is suggested that the first peak in the respiration rate of the older bees occurred at the lowest temperature at which free movements were possible, and that it indicated an increased body temperature maintained by the bees to combat the effects of the low environmental temperature. The lack of this first peak in the case of the youngest bees is thought to mean that they were unable to raise their temperature above that of the environment. The information obtainable from the literature supported these conclusions; their implications in relation to the regulation of temperature within the colony are discussed.

5. Numerical values for the oxygen consumptions of adult bees are discussed in relation to those found by other workers.

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# UNIDIRECTIONAL MOVEMENT FIBRES FROM A PROPRIOCEPTIVE ORGAN OF THE CRAB, *CARCINUS MAENAS*

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Proprioception in the legs of arthropods arises in receptor cells more closely associated with structures of the joints than with muscles. Sensitive endings are located in special internally placed organs (Barth, 1934; Burke, 1954; Alexandrowicz & Whitear, 1957). Studies of the action potentials in the leg nerve during mechanical manipulation of the leg joints in *Limulus* (Pringle, 1956) and in certain arachnids (Pringle, 1955) have shown the presence of several types of proprioceptive endings. Slow-adapting tonic position fibres, some responding at the flexed and others at the extended positions, were shown to have their endings situated so as to react to strains in the cuticle. The rapidly adapting phasic fibres, however, originate in some internal structures, different fibres responding to the two directions of movement.

Burke (1954) described and studied an internal organ present in the joint between the dactylopodite and propodite of *Carcinus maenas*, the shore crab. Phasic responses were initiated during movement in either direction, and resting discharges continued when the claw was held in the open or closed positions. This organ, the pro-dactylopodite (P.D.) organ, consists of a stretched elastic strand and associated nerve cells. It is attached at one end to the inner surface of the dactylopodite and at the other to an apodeme, functioning as the tendon for the closer muscle. As this tendon is moved by the muscle during closing (flexion), the elastic strand is further stretched, increasing its length by about 1 mm. As this structure signals both position and movement, it is important to know how many different types of endings are present: and also whether a single organ with such an apparently simple structure can completely analyse both directions of movement, and in addition signal the degree of both flexion and extension. The single-fibre technique was therefore employed to determine the types of sensory axons making up the P.D. nerve bundle of *Carcinus*.

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In most experiments the organ was left *in situ* in order to avoid the changes in excitability resulting from interference with its suspensions when dissections are performed near it. The experimental set-up employed is illustrated in Fig. 1. The walking legs of *Carcinus maenas* were used throughout. The nerve was exposed in the meropodite by removing the inner half of the surrounding shell and all the muscles. The nerve was first split with fine needles into the naturally occurring bundles. Bundles not reacting to manipulation of the joint were discarded, whereas any active bundle was kept. In this way a single thin naturally occurring bundle

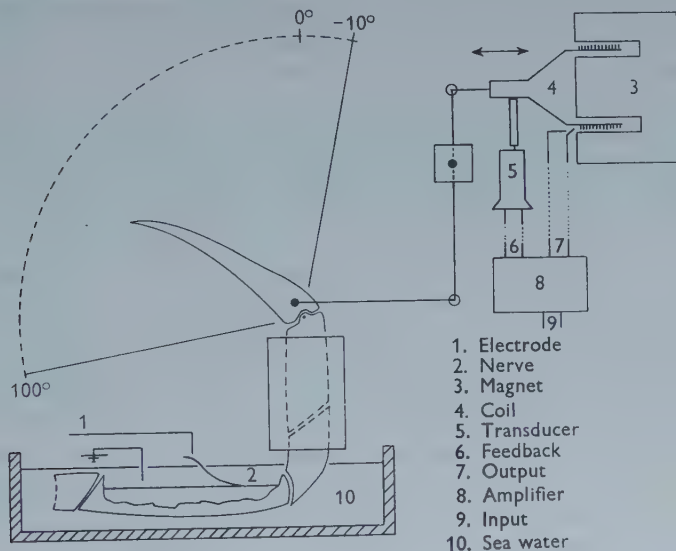


Fig. 1. Drawing showing preparation and experimental apparatus.

was regularly found, and subsequently further divided by the technique previously described for obtaining single active efferent fibres (Wiersma, 1941). *Carcinus* proved to be highly suitable for this purpose, as splitting of the fine subbundles could be more readily performed than in most other species. Splitting in either direction was usually successful. In most other species, unless splits are made from the periphery, connective tissue strands are liable to constrict the fibres, in *Carcinus* these strands are weak. Larger fibres could be used singly, but small ones have so much electrical resistance and dry out so quickly that in isolation they are not suitable for the leading off methods employed. Instead, small bundles of such fibres were prepared and used if the few reacting fibres in them gave response patterns which could be distinguished with certainty.

The nerve was kept just below the level of the sea water in which the preparation was submerged. After a small bundle was obtained in which the polyphasic lead showed the presence of a promising fibre, the lead was made monophasic by lifting the little bundle out of the fluid with the active electrode and letting it dry out. The

other electrode remained in the water and was connected to earth. Under these circumstances, the place where the bundle breaks through the surface acts as the active electrode. The action potential spikes were amplified by a Tectronic 112 preamplifier, the output of this being fed into a Cossor oscilloscope and a loud speaker.

The receptor organ was mechanically stimulated by moving the dactylopodite passively by means of the coil of a permanent magnetic loudspeaker unit, which functioned as an element of a servomechanism activated by voltage changes. The response of the unit was very rapid and non-oscillatory. Extremely smooth movements were achieved by feedback control of the heavily damped coil. Ordinarily, movements with a constant velocity were used in this investigation. These were produced by obtaining the activating voltage change from a Hewlett-Packard function-generator capable of producing linear voltage changes which caused less than a  $1^\circ/\text{sec.}$  movement of the dactylopodite.

The actual movement of the coil, picked up by a transducer which furnished the negative feedback, was also used to drive the second beam of the oscilloscope. The movement recorded on the films was therefore that of the coil and not of the dactylopodite. The latter's movement followed that of the coil very closely, in spite of a slight unavoidable play in the lever system linking them and of the small error introduced by converting linear to angular motion.

## RESULTS

Confirming Burke's findings (1954), a specific bundle of large and small fibres responsive to manipulation of the dactylopodite could readily be isolated in the meropodite. This bundle, usually located adjacent to the closer motor axons, proved to contain all the fibres giving proprioceptive discharges from the P.D. organ. In some instances responses to movement were found in other bundles, but on closer observation these were shown to arise from different sources. Among these were hairs on the dactylopodite and propodite, and proprioceptors of other joints of the leg, which come readily into play unless special precautions are taken. Using a large clamp and moving the dactylopodite by means of a pin inserted into its base, stimulation of other receptors could be avoided. Under these conditions, all fibres reacting to displacement of the P.D. joint were found to be restricted to the P.D. bundle. As will be shown later, not all fibres in the P.D. bundle arise from the P.D. organ itself, but such fibres did not play a part in the responses to be described (see below).

## DESCRIPTION OF THE FIBRE RESPONSES

The unit responses obtained show clearly that the reacting sensory cells can be divided into a number of response types. In the first place, about half of the fibres respond to opening (extension of the dactylopodite), whereas the others respond to closing (flexion). In each of these classes, fibres are present which discharge only during the movement of the dactylopodite, and others which continue to fire when the joint is near one of the extreme positions. Among the fibres responsive only

during movements, none were found that respond in any part of the arc to both opening and closing.

A. *Position fibres*. Occasionally fibres are found which approach closely the ideal responsiveness of a structure designed solely for signalling position. Such a receptor should have no adaptation and should be unaffected by movement. Fig. 2*a* illustrates the responses of two such fibres present in a small bundle. On moving the dactylopodite from  $-5$  to  $-10^\circ$  (see Fig. 1) there is no burst of impulses in either fibre during movement, but in both the frequency increased markedly with changing position and was maintained during the remainder of the record. Indeed, such fibres will continue to fire, with little change in frequency, for very long periods. As with many fibres of this type, these two are sensitive to position over only a small part of the total arc. They both stopped discharging at  $0^\circ$ .



Fig. 2. Position fibres. Upper line indicates movement of the dactylus, down opening; up closing. Lower line shows action potentials. (a) 'Pure' position fibre; (b) position fibre showing different sensitivity at the same position attained during opening and closing.

Commonly encountered are fibres in which adaptation is more pronounced, though a tonic discharge is maintained in the most extreme position. These are usually larger than the more ideal type and so are easier to find. In Fig. 2*b* the response of such a fibre is shown when the dactylopodite was moved toward and away from the extreme opener position. The fibre starts to discharge at  $10^\circ$  but stops on the return at  $5^\circ$ . The whole discharge is markedly asymmetrical, there being many more impulses during opening than during closing. The behaviour of this type of fibre does not need any special discussion, since it is similar to that of the ligament organ in vertebrates (Boyd & Roberts, 1953).

Except for those signalling the more extreme positions, position fibres are difficult to prepare by the methods employed. It is therefore uncertain whether the complete range of position is covered. Most position fibres are of small diameter, and failure to find them may be due either to their size or to their rarity. Certain fibres, responding tonically to one of the extreme positions, are also brought into activity by rapid movement and are discussed below.

B. *Unidirectional movement fibres*. Some of these fibres, which are encountered in every preparation, discharge on the slightest movement, provided it is in the

direction for which the ending is sensitive. The threshold of the most sensitive opening movement fibres is lower than for the most sensitive closing fibres. Sensitive opening movement fibres begin to fire at movement velocities of less than  $1^\circ/\text{sec.}$ , at which speed it takes 2 min. to open fully from the closed position ( $110^\circ$ ,

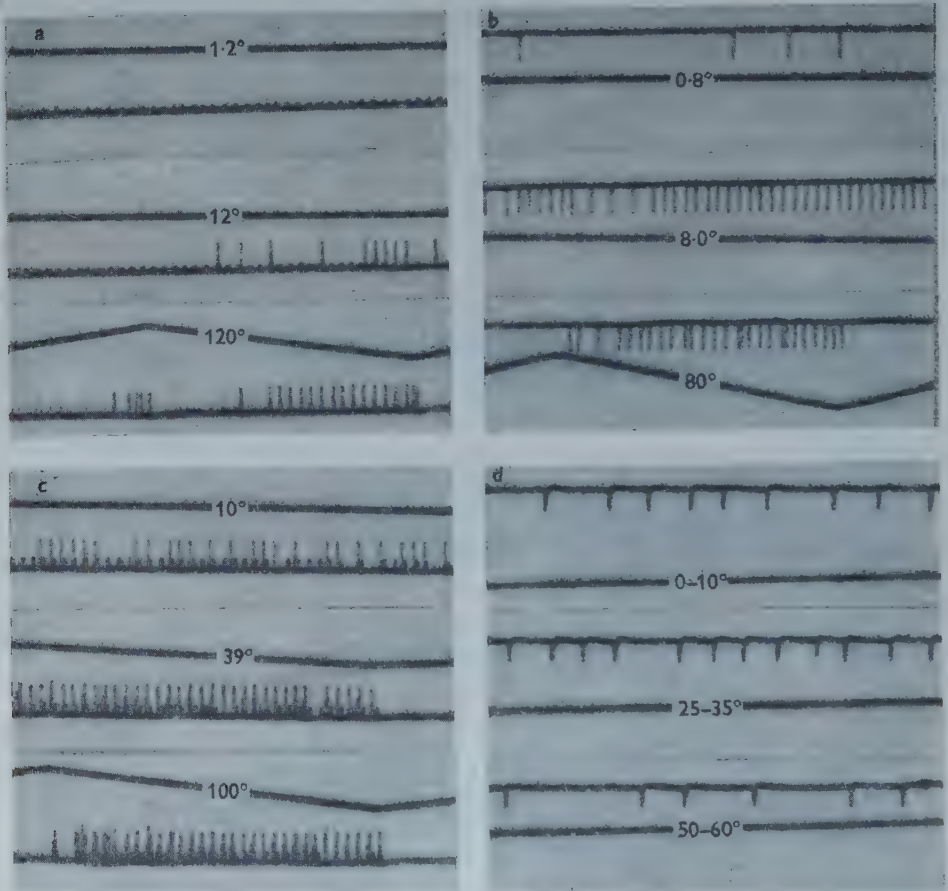


Fig. 3. Movement fibres. Upper line indicates movement of the dactylopodite, down opening; up closing. Lower line shows action potentials. In (a), (b) and (c) the figures on the records show the speed of movement in degrees per sec. In (d) the figures give the movement position arc in degrees, the velocity being constant at  $1^\circ/\text{sec.}$  (a) A very sensitive opening fibre, a less sensitive opening fibre, and a very insensitive closing fibre firing only at the end of the movement. (b) A sensitive opening fibre showing double firing at the fastest speed. (c) Three opening fibres with different sensitivities. (d) A sensitive closing fibre showing response at different portions of the movement arc at near threshold velocity.

see Fig. 3). Their responses are very nearly those of a pure unidirectional movement fibre, differing from the ideal, near threshold and at high velocities, in two ways. In the first place, at threshold they are not completely independent of position. In general, the opening fibres are less sensitive to movement near the closed position

and the closing fibres near the opened position. However, for the rest of the movement they are as responsive in one part of the arc as in another. Secondly, the movement fibres are not quite independent of the velocity of movement. Near threshold speeds the spikes appear very irregularly, but while the movement velocity is still very low a maximum frequency is attained over the whole arc. Thus in Fig. 3*b* there is no significant increase in frequency though the speed increases from 8 to 80°/sec. The small fibre shown in Fig. 3*a* was one of the most sensitive found. It attained full saturation, about 40 impulses/sec., at 1.2°/sec. and showed no change in frequency to 120°/sec. (complete opening movement in 1 sec.). Even at speeds three or four times as fast, the same signal could be detected without change in frequency, though partially obscured by the signals of the other fibres. Such fibres cannot play a significant role in mediating the velocity of movement, but only in mediating the movement itself and its direction.

In Fig. 3*d* the response of a sensitive closing movement fibre is shown near threshold, the three records being from different parts of the movement arc as indicated. Some increase in frequency is evident near the mid-position and the firing is quite irregular. The response of a small bundle of opening movement fibres is shown in Fig. 3*c*. The smallest response is that of the most sensitive fibre, which is saturated at the slowest speed shown. The largest response is of a fibre with a higher threshold, which is not fully saturated at this speed but becomes so at 39°/sec. The fibre with intermediate spike height is the most insensitive and does not fire at the slowest speed. At 39°/sec. it responds in an irregular manner and by 100°/sec. fires regularly. In all these fibres, though they have such very different thresholds, the range of velocities through which the discharge frequency increases is thus limited, and each by itself is a poor indicator for velocity.

Though there was no graded increase in frequency with increase in speed in any of these fibres, some have a strong tendency to give more and more double or even triple discharges at high velocities of movement. Multiple responses are illustrated in Fig. 3*b* and *c* at the fastest speeds.

Rather regularly encountered among the large movement fibres are some responsive at threshold speeds only to movement at one side or the other of the mid-position. Occasionally one was found that fired only at the beginning of a full opening movement if the fibre was an opening fibre, or at the beginning of closure if the fibre was a closing fibre. Such behaviour is difficult to explain and may not be normal. Usually opening fibres of this type are most sensitive near the opened position and closing fibres near the closed position. Frequency of discharge is more dependent on both velocity and the degree of opening (or closing) than in the sensitive movement fibres. Such an opening fibre will have a lower threshold near the opened position, and the further from the opened position the greater will be the movement speed required to produce any given discharge frequency. Toward the closure side the fibre may not fire at the fastest speeds used, though certainly some can be made to respond in any region. The large spikes in Fig. 3*a* are from two different fibres, an opening and a closing, firing only after the movement has passed the mid-position. At 12°/sec. the opening fibre begins to fire and at 120°/sec. its

discharge has become regular but limited to about one-half of the arc. The closing fibre shows only five spikes at the end of closure at  $120^\circ/\text{sec}$ . Fibres with the larger spikes tend to adapt to the movement stimulus more readily and the largest ones may show a greater increase in frequency than those illustrated; but such fibres were rarely found.

There appears to be a complete gradation from fibres responding as nearly pure movement fibres, affected little by velocity or position, to those whose discharge frequency is affected by both to various degrees. Fibres of the latter type are commonly used by vertebrates for signalling proprioceptive information, while specialization toward the development of pure unidirectional movement fibres has not previously been reported.

Some fibres behave as rapidly adapting unidirectional movement fibres over part or all of the movement arc, and as position fibres when the dactylopodite is at the end of its movement, fully opened for opener fibres and fully closed for closure fibres. This type has been found especially in other species of Crustacea (Wiersma, 1959). With respect to the classification used here these fibres are intermediate.

#### LOCALIZATION OF CELLS RESPONSIBLE FOR DIFFERENT FIBRE RESPONSES

Preparations of the P.D. organ were made by opening the propodite on the lateral posterior side. By removing the muscle fibres of the closer muscle on that side, the organ can be readily exposed with the nerve bundle going to it. These preparations were used for physiological experimentation and subsequent staining with Rongalit methylene blue (see Fig. 4). On the whole, our results were similar to those of Burke with respect to the anatomical relations.

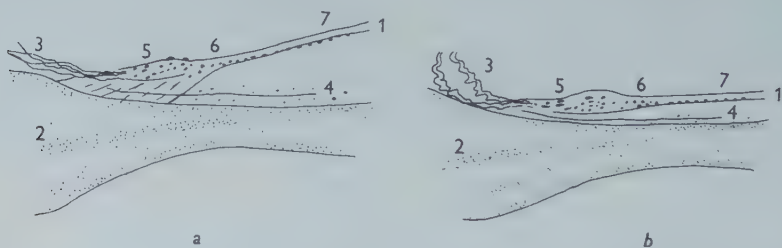


Fig. 4. Drawing of the P.D. organ showing the orientation of the elastic strand and the positions of the cells referred to in the text. (1) Elastic strand. (2) Tendon. (3) P.D. bundle. (4) Tendon nerve branch. (5) Proximal cell group. (6) Distal cell group. (7) Line of small cells extending up the strand. (a) Opened position; (b) closed position.

Large cells are clustered at the tendon end of the organ and small cells extend along a good part of the elastic strand. It was found that a branch of the nerve bundle leading to the organ leaves the main part of the bundle to go to the distal part of the tendon. In some cases, one or two nerve cells could be discerned, but the further course of the fibres was not revealed.

The branch can be split off rather readily from the main bundle, and three types of experiments were performed in order to establish the role, if any, that this branch plays in the response on movement of the dactylopodite. (a) In preparations in which the main bundle gave clear responses of all types the tendon branch was invariably silent. (b) Cutting the tendon branch between a point near the closer tendon and its junction with the point where it joins the main bundle did not alter the response obtained from the P.D. bundle in any noticeable way. More specifically, all four main types of responses were still present. (c) Cutting the branches going to the P.D. organ, but leaving the tendon branch, resulted in every instance in the disappearance of all responses in the P.D. bundle to manipulation of the joint. As to the kind of stimulus mediated by the tendon branch we have no experimental evidence.

In the main bundle one can readily detect responses of different kinds. But the exposed organ is much more sensitive to outside disturbances such as water movements. Hence it is not possible to distinguish the responses with the same precision as in the meropodite preparation. The reason for using this preparation was that it is possible to split the bundle into several subbundles which can be traced to their origin. Especially in the closed position, the nerve near P.D. is coiled and can be seen to consist of several bundles (see Fig. 4*b*). It was found possible to split these naturally occurring divisions further until enough length of nerve for leading-off purposes was obtained.

There are often two main subbundles each coming from a different side of the organ. A successful split brought about the separation of opening and closing fibres. The bundle containing the closing fibres showed often a peripheral and a central branch. A further split revealed that the peripheral branch contained many position fibres, the central one preponderantly movement fibres. For the other subbundle a similar division of opening movement fibres and opening position fibres was attained rather clearly in a single experiment only. Further evidence is necessary to establish this localization of function.

By tracing the subbundles which reacted predominantly to one type of stimulation it was found that opening fibres originated from a cluster of cells, some large and others small, located near the tendon end of the organ but rather on the side away from the tendon (see Fig. 4(5)). The closing subbundle was traced to another cluster not quite so loosely attached to the elastic strand and situated somewhat further distal and more on the tendon side (see Fig. 4(6)). As stated, when a peripheral bundle could be prepared it contained position fibres and could be traced to the small cells which extend along the elastic strand towards its insertion on the dactylopodite (see Fig. 4(7)).

#### DISCUSSION

In view of the structure of the P.D. organ, the most surprising result of this study is the strictly unidirectional nature of the individual endings of the different sense cells, whereas the organ as a whole has similar sensitivity for both directions. The separation of the endings sensitive to flexion from those sensitive to extension must

be related to the mechanical stresses to which the two types of endings are subjected. How this can be brought about is therefore an important question to which there is, as yet, no certain answer. As was shown by Burke (1954), the elastic strand in which the nerve endings appear to be embedded is under tension in all positions of the dactylopodite. It can be observed that, on closing, the tendon moves in such a direction as to increase the tension in the elastic strand. At the same time the angle between the tendon and the elastic strand decreases from  $17$  to  $7^\circ$ , a change of  $10^\circ$ , due partly to the movement of the tendon and partly to rotation of the dactylopodite which moves the insertion of the elastic strand (see Fig. 4). The presence of linear as well as angular movements—which are of opposite phase, the angle being smallest when the linear change is largest—suggests a possible explanation. Many of the fibres of the elastic strand attach to the tendon at points which are located as a straight continuation of the strand itself. But others, rather thin and more difficult to see, run to the tendon across the angle between the strand and the tendon. During closure, when tension in the main strand increases, the tension in the latter elastic fibres would decrease, since the angle is reduced. One would, therefore, expect to find that the group of nerve cells shown to be associated with opening have their endings associated with the elastic fibres fanning out from the strand towards the tendon, whereas the group of closure cells should be more closely associated with the main strand of elastic fibres.

In our methylene-blue stained preparations no final decision on this important point could be reached. The opening movement cells were closer to the point where the strand attaches to the tendon and were rather loosely attached to it. However, they lie generally more on the side of the organ away from the fanning elastic fibres. Certain stained preparations were photographed with the organ in extreme opened and closed positions (see Fig. 4*a, b*). In these it could be seen that the cell positions alter considerably, showing that indeed different cell groups are exposed to different displacements and forces. It will be shown in another paper (Wiersma, 1959) that in other joint organs the stimulation of most of the sense cells comes about when the tension in the main elastic strand is diminished. Further studies are required to show the relations between the nerve endings and the elastic fibres, and what constitutes the stimulating factor. Although the nerve endings were not studied in detail it was noted that the dendrites of the large bipolar cells at the proximal end of the organ appeared as spirals.

It has been pointed out that individual movement fibres are usually poor velocity fibres. By the recruitment of several fibres with different sensitivities during faster movements the C.N.S. could, of course, extract this information. When the different types of position fibres are also considered it is evident that enough information is transmitted to allow a very precise analysis of any peripheral change in the joint, the only possible exception being that there is no information around the mid-position when the joint is kept perfectly still. In contrast with Burke, we observed a number of instances in which no fibres were observed to fire under these conditions, but the possibility that very small fibres would regularly do so is not completely excluded.

From our observations, Burke's thesis that the organ is used to detect vibrations appears unlikely. We have confirmed his observation that the isolated organ is very sensitive to vibrations, but have found that the responses to movements as small as he used occurred in the most sensitive unidirectional movement fibres, and in these only during the appropriate phase of the movement. Consequently, these fibres could not distinguish between a vibration and a continuous movement. In experiments in which the dactylopodite was vibrated at various amplitudes up to 100 cyc./sec. the very small movements were always detected only in the sensitive movement fibres. As the amplitude was increased, velocity increasing as well, the larger rapidly adapting fibres responded during the phase of the movement appropriate to their sensitivity. In these fibres one-to-one responses were found up to 100 cyc./sec., whereas the sensitive fibres do not usually discharge above 40 impulses/sec.

There seems little doubt that the development of sensory endings mediating unidirectional movement nearly independent of position or velocity, is connected with the presence of an exoskeleton and the absence of sense organs in the muscles. In animals with an internal skeleton similar endings could occur in the joints. It is interesting to note that movement fibres have been reported in the knee joint of the cat, though these are not unidirectional (Boyd & Roberts, 1953).

The authors wish to express their appreciation to members of the staff of the Zoological Laboratory, Cambridge, for providing facilities and equipment necessary for this investigation and for inviting us to work in the Zoological Laboratory during the tenure of our Fellowships.

#### SUMMARY

1. The proprioceptive organ of the shore crab *Carcinus maenas*, which signals all proprioceptive sensation from the joint between propodite and dactylopodite, has been shown to contain sense cells receptive to position as well as to movement.
2. Certain cells of the organ increase their discharge frequency in response to greater degrees of flexion, while other endings respond to greater degrees of extension. Movement has little effect on the frequency of the position fibres.
3. Cells responding to movement in one direction only are present, certain ones signalling flexion and others extension. The most sensitive unidirectional movement fibres are almost completely independent of position and velocity. They provide the crab with an extremely sensitive movement sense not as yet reported in other proprioceptive mechanisms.
4. Both position-sensitive and movement-sensitive cells show a wide range of thresholds.
5. Cells which cannot be strictly classified as either position or movement sensitive have also been found.
6. By tracing the origin of the signals to the cell bodies, it appears that movement fibres have on the whole larger cell bodies than position fibres and are more proximally located.

7. Flexor and extensor movement cells appear to lie on different sides of elastic strand.
8. The question of how unidirectional sensitivity may be achieved is discussed.

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# THE COMPOSITION OF THE BLOOD OF THE SHORE CRAB, *CARCINUS MOENAS* PENNANT, IN RELATION TO SEX AND BODY SIZE

## I. BLOOD CONDUCTIVITY AND FREEZING-POINT DEPRESSIONS

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### INTRODUCTION

Although the literature on the ionic and osmotic regulation of crustacea is extensive (reviewed by Krogh, 1939; Prosser *et al.* 1950; Beadle, 1957), in only a few cases has any reference been made to the sex and body size of the animals examined. This is surprising, for it is well known that both of these factors have a profound influence on the general metabolism of animals: their influence on the ionic content and total osmotic pressure (O.P.) of the blood of *Carcinus moenas* is investigated in the present work.

### MATERIALS AND METHODS

Crabs were obtained each week from the shore close to the Dove Marine Station, Cullercoats. They arrived in this laboratory within a few hours of being collected. Berried females and injured animals were discarded, and the remainder kept in lots of twelve in aquaria containing 40 l. of well-aerated Cullercoats sea water. Under these conditions the crabs remained alive indefinitely. The animals were allowed to remain in the tanks for at least 24 hr., but prior to the experiments they were transferred in lots of three to similar aquaria, where they remained for 48 hr. in order to reach complete ionic and osmotic equilibrium (Margaria, 1931). Since Bateman (1933) has shown that the vapour pressure of the blood of *Carcinus* remains constant over the temperature range of 2.3–15.7° C. the aquaria were maintained between 7–11° C. by mains water circulating through coils of polythene tubing.

The animals were not fed while in the laboratory and it was hoped that variations in their blood composition due to differences in feeding would thus be eliminated.

The experiments were begun in 1953, and since it was clear that they would extend over a considerable time they were designed so as to reduce any seasonal effects which might be operating. On any one day the crabs were chosen from the widest size-range possible, including at least one of each sex from the following arbitrary size groups, 0–15, 30–45, 60–75 g. weight. The whole size range was

therefore continually covered and any seasonal effect would thus apply equally over each part of the range.

Since the blood composition varies during the moult cycle (Baumberger & Olmstead, 1928), and since particularly just prior to the moult there is a considerable increase in the calcium content of the blood (Robertson 1937), measurements of total O.P. and conductivity were confined to animals in the intermoult stage. This was achieved by discarding crabs obviously about to moult and by only using blood that was pale and clear, since towards the end of the intermoult stage there is an accumulation of red pigment present in the blood.

Crabs were removed from the aquaria, lightly blotted to remove surplus water and weighed to the nearest 0.5 g. on a Tower's sliding weight balance. Carapace lengths and widths were then measured with vernier callipers.

Blood was removed through a small incision in the arthrodial membrane at the base of the large chela. Using a glass pipette and rubber teat it was possible to obtain sufficient blood from a single crab for both the conductivity and total O.P. determinations. For the O.P. determinations the blood was placed in heparinized tubes to prevent clotting: this was unnecessary for the conductivity measurements since, by reason of the low resistance of whole blood, it had to be used diluted.

A small measured volume of about 0.06–0.07 ml. was removed with a standardized Pyrex capillary pipette from 10 ml. of distilled water; this was replaced with an equal volume of blood, thus diluting the blood about 150-fold. The conductivity was then determined with an EEL conductance bridge and, since dilute blood was used, the conductivity value was compared with that for a standard sodium chloride solution.

There were slight variations in the temperature of the laboratory during the course of the work; this would affect the conductivity values. However, since the whole size range of both sexes was covered each day, this would only influence the variation in the data, but not the essentially comparative nature of the present work.

#### FREEZING-POINT DEPRESSIONS

Freezing-points of 1 ml. samples of heparinized blood were determined by the method of Johlin (1931); the freezing-bath being stirred by hand. Three determinations were made on each sample with a Beckman thermometer reading to  $\pm 0.003^{\circ}$  C. and the mean evaluated. Measurements with distilled water were carried out each day to correct for fluctuations in the thermometer zero.

The quantity of heparin used had a barely detectable effect on O.P.

#### RESULTS

In Fig. 1 carapace width has been plotted against body weight on a double logarithmic grid for 101 female crabs varying in weight from 2.75 to 61.5 g. The relationship between carapace width and body weight remains constant. This was also found to be true for the males and for the relationship between body weight

and carapace length of both sexes (see also Shen, 1935). Any of these measurements could therefore be used as a standard of body size. For convenience body weight has been used throughout this work.

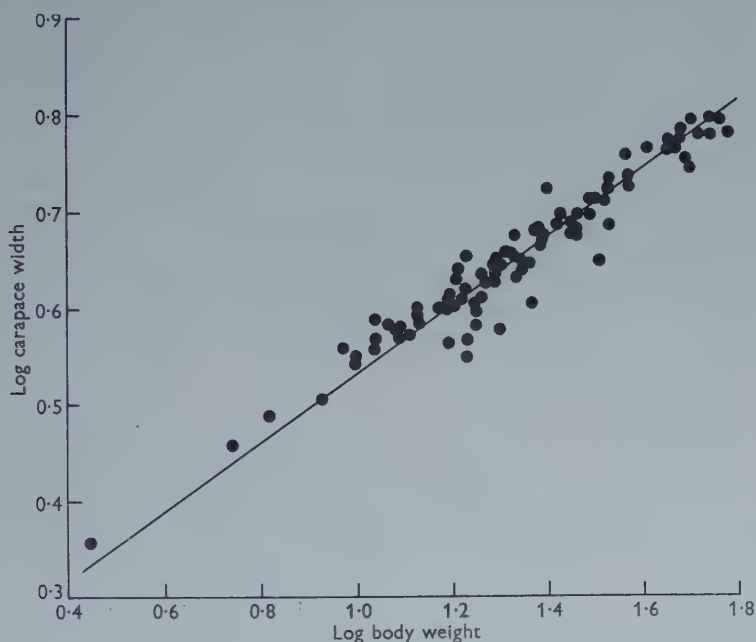


Fig. 1. Carapace width plotted against body weight on a double logarithmic grid for female crabs.

### CONDUCTIVITY

Conductivity values expressed as grams of sodium chloride per litre have been plotted against body weight for 81 male crabs in Fig. 2 and 106 females in Fig. 3. Despite the wide scatter of the results, it is clear that in both cases the conductivity is not constant over the whole size range. From a value equivalent to about 31 g. NaCl/l. for the smallest animals the conductivity rises steadily and reaches a maximum value of about 33 g. NaCl/l. for crabs of 35 g. body weight. After this there is a slower decrease in the conductivity values until they reach about 31.5 g. NaCl/l. for the largest crabs used.

For the statistical analysis the data for each sex was divided into two groups, viz. for those animals over 35 g. body weight and for those animals less than 35 g., and the respective regression coefficients calculated by the method of least squares. Although this treatment is somewhat arbitrary, reference to Figs. 2 and 3 will show that it is not unjustified. As would be expected from the figures, all four regression lines, one each for the males and females below 35 g. body weight, and one each for the males and females above 35 g. body weight, differ significantly from the horizontal ( $P < 0.01$  in each case). Moreover, covariance analysis showed that the two regression coefficients for the lines of positive slope for male and female crabs

below 35 g. body weight do not differ significantly from each other, nor do the two regression coefficients for the lines of negative slope for males and females above 35 g. body weight ( $P > 0.05$  in both cases). There was also no difference in the

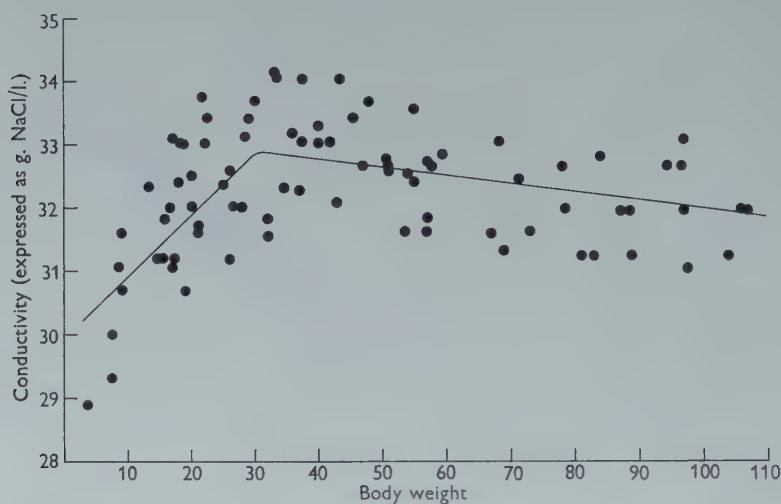


Fig. 2. The relationship between conductivity (expressed as g. NaCl/l.) and body weight for male crabs.

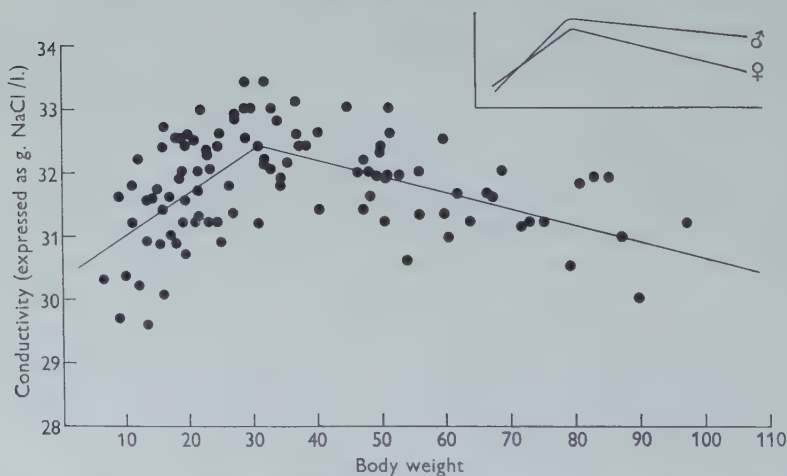


Fig. 3. The relationship between conductivity (expressed as g. NaCl/l.) and body weight for females. Inset: The calculated regression lines taken from Figs. 2 and 3 plotted together.

conductivity for male and female crabs below 35 g. body weight ( $P < 0.05$ ). However, in the case of animals over 35 g. body weight the conductivity of the blood of the males tended to be higher than that of the females: this difference is highly significant ( $P < 0.01$ ).

## FREEZING-POINT DEPRESSIONS

For convenience, values of the freezing-point depressions were converted to a millimolar basis using the relationship that a molar concentration of a non-electrolyte has a freezing-point depression of  $1.86^{\circ}\text{C}$ . These values have been plotted against body weight in Fig. 4 for forty-one males and Fig. 5 for thirty-nine female crabs. In both sexes there is a marked tendency for the values to decrease with increasing body weight, the regression coefficients being  $-0.283$  and  $-0.202$  for the males and females, respectively. With standard errors of  $\pm 0.101$  and  $\pm 0.061$ , respectively, both lines differ significantly from the horizontal ( $P < 0.01$  in both cases). There is no significant difference between the regression coefficients for the two sexes ( $P > 0.05$ ). However, the O.P. of the males tended to be significantly higher than that of the females over the whole size range; covariance analysis shows that this difference is highly significant ( $P < 0.01$ ).

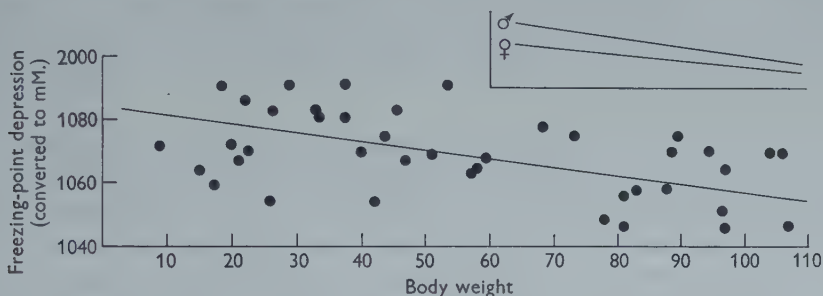


Fig. 4. The relationship between freezing-point depressions of the blood (converted to a millimole basis) and body weight for males. Inset: The calculated regression lines taken from Figs. 4 and 5 plotted together.

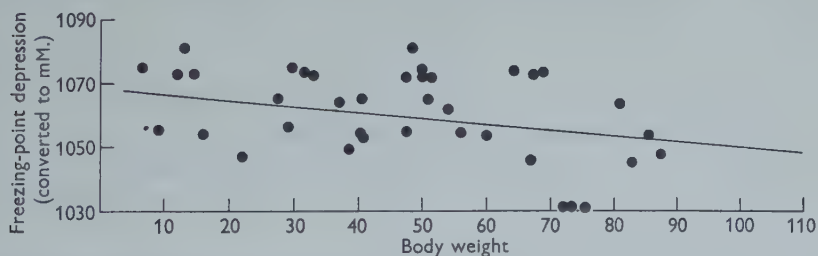


Fig. 5. The relationship between the freezing-point depressions of the blood (converted to a millimole basis) and body weight for female crabs.

## DISCUSSION

It has been shown that the blood composition of the common shore crab varies in the following ways. The O.P. decreases with increasing body weight in both sexes; the male tending to have a higher O.P. than the female. In both sexes the conductivity of the blood of the smallest and the larger crabs is lower than that of crabs of 35 g. body weight. For animals over 35 g. body weight the conductivity values for the blood of the males are significantly higher than those of the females.

These differences in the O.P. of the blood may resolve the controversy as to whether *Carcinus* is hypotonic, isotonic or hypertonic (Duval, 1925; Nagel, 1934; Schlieper, 1929; Picken, 1936), since the majority of these workers did not record the size or the sex of the animals they used. Moreover, observations of Panikkar (1941) on *Leander* suggest that a variation of total O.P. may not be true of *Carcinus* alone.

Clearly, crabs of different size and sex must have different ionic and osmotic relationships with the environment. In the case of the small males the blood is hypertonic, and in the large crabs hypotonic, to the environment, while the females are hypotonic over the whole size range. This may be of importance for the life of a littoral and estuarine animal, and of ecological significance.

Since the conductivity of a solution depends on the charge and the mobility of each ion present, the differences in the blood conductivity could be due either to a variation in the total ions or to a change in the relative proportions of the different ions. Although the observations on the O.P. suggest that the latter is more likely to be correct, the O.P. of the blood is not entirely dependent on the ionic content, and as will be shown in due course the non-electrolyte fraction cannot be disregarded.

It is becoming increasingly apparent that body size is of great importance in zoological investigations. Its effect on oxygen consumption is well known, and recently Parry (1958) has shown that the ability of salmonid fish to withstand dilute sea water is related to body size. Furthermore, both Panikkar (1941) and Bogucki (ref. Beadle 1957) have suggested that some size effect may be operating on the osmotic regulation of *Leander* and *Nereis*, respectively. Since size is related to age, the observation of Beadle (1957) on the 'embryology' of osmotic and ionic regulation therefore becomes increasingly significant.

#### SUMMARY

1. The influence of body size and sex on the total osmotic pressure (O.P.) and blood conductivity of the shore crab was investigated.
2. In both sexes the O.P. fell steadily as body weight increased.
3. At any body weight the O.P. of the blood of male crabs was significantly higher than that of females.
4. Blood conductivity increased in both sexes until a maximum was reached at a weight of about 35 g. Thereafter the conductivity fell as the weight increased.
5. There was no significant difference in blood conductivity between male and female crabs below 35 g. body weight. Above 35 g. the conductivity of males was significantly higher than that of females.

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# SEASONAL VARIATION IN THE ACTIVITY OF THE THYROID GLAND OF YEARLING BROWN TROUT *SALMO TRUTTA* LINN.

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(Received 12 September 1958)

(With Plate 3)

## INTRODUCTION

In a previous paper (Swift 1955) the seasonal variation in the activity of the thyroid of 3-year-old brown trout, determined by the rate of loss of radio-iodine from the gland, was described. It was noted that this cycle of activity differed from that described in yearling trout by Smith (1956). Smith using histological criteria found no evidence of a summer activity peak, whereas the radio-iodine technique showed that this was the period of maximum glandular activity in the older fish. It was felt important to find out the reason for this discrepancy which could have arisen either as a result of the age difference between the two lots of fish examined, or because the summer activity was not manifest in the histological appearance of the gland. The results reported in this paper are from a histological and radio-iodine examination of the thyroids of samples of fish from one population or yearling trout.

## METHODS

A population of yearling trout were reserved for this work in a large stewpond at the experimental hatchery of the Freshwater Biological Association; they received the same treatment as normal hatchery stock, and at sampling time random samples of thirty-two fish were taken for examination.

## RADIO IODINE

The technique used for the determination of the glandular activity with radio-iodine was exactly the same as that previously described (Swift, 1955). After injection with isotope solution the activity of the thyroid area was determined at intervals of 24, 48, 72 and 96 hr. The fish, anaesthetized with tricaine methano sulphonate were allowed to recover in running water after each examination and returned to the hatchery. The regression coefficient for the slope of the iodine loss time curve over the period of examination was taken as the index of activity of the gland, all counts being corrected for background count and the natural decay of the isotope.

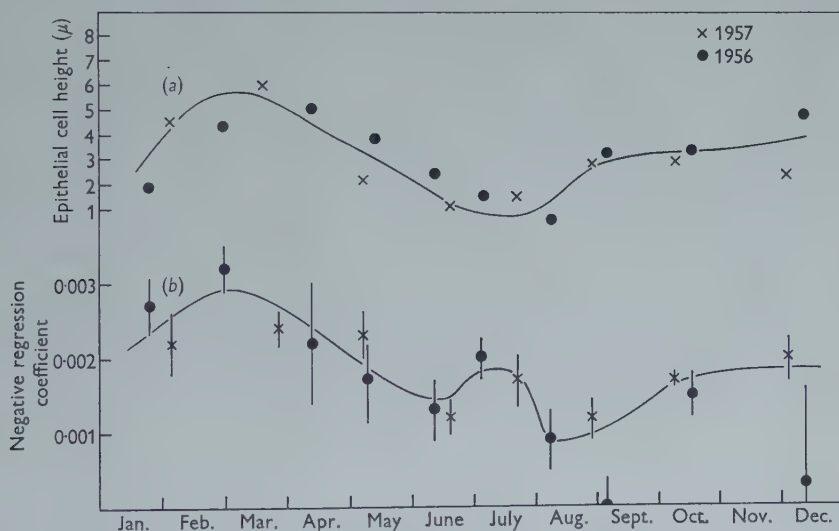
From the sample of fish, twenty were examined with radio-iodine, and the remaining twelve were used for histological examination.

# HISTOLOGY

The fish were killed by a blow on the head and the thyroid area was immediately excised and fixed in corrosive formaldehyde. The fixed tissue was dehydrated in diethylene dioxide and embedded in ester wax. Sections  $10\ \mu$  thick were cut and stained in Delafield's haematoxylin and eosin Y. The sections were examined at a magnification of  $\times 500$  and the epithelial cell height of thirty follicles from each fish was measured. Thirty follicles were examined, as this was found by experience to be the largest number of follicles visible in one section of the thyroid for all the fish in the period under review.

The temperature of the water in which the fish were living was taken evening and morning and the results expressed as an average, calculated to each sampling time.

This work was first carried out during 1956, and was then repeated during 1957 using a fresh population of yearling fish.



Text-fig. 1a. Seasonal variations in the thyroid epithelial cell heights during 1956 and 1957. Each point represents the mean height of thirty follicles from twelve fish. In each case twice the standard deviation from the mean is too small to show ( $< 3\%$ ).

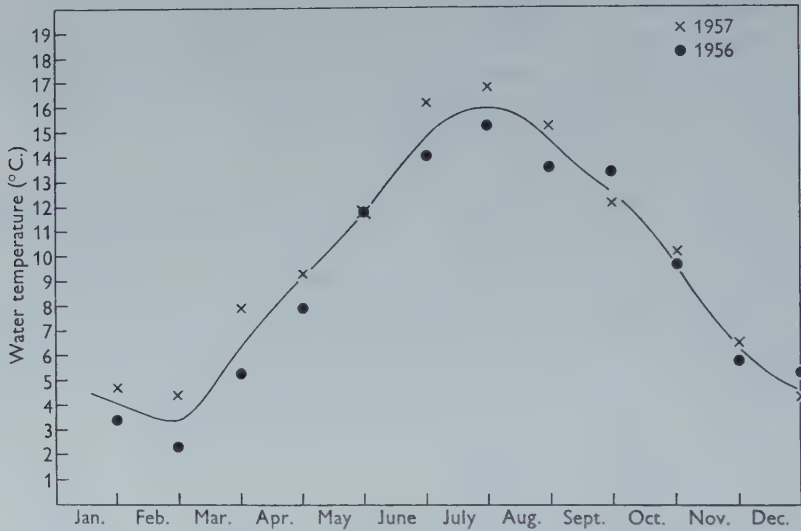
Text-fig. 1b. Seasonal variations in the thyroid gland activity during 1956 and 1957. The glandular activity is expressed by the regression coefficient for the iodine loss/time curve. (The height of the vertical lines  $= 2 \times \pm$  the standard error of the mean.

# RESULTS

The mean epithelial cell height for each batch of fish is shown in Text-fig. 1a. The results for both years 1956 and 1957 are so similar that they have been combined on one graph and one trend curve drawn through the points. The picture of the seasonal variations in glandular activity agrees with the findings of Smith and shows a maximum activity in February and March, then a slow decline to July, after which

the activity after an initial sharp rise during August slowly increases throughout the autumn and winter.

The results recorded with the radio-iodine technique are shown in Text-fig. 1*b*; again the results were so similar for the 2 years that they are combined in the one graph. This method confirms that the peak thyroid activity occurs during February declining to June, but then demonstrates that, as was found in the older fish (Swift, 1955), the activity of the gland rises sharply during June, falling again during July to reach its lowest level in August, after which it slowly rises during the autumn and winter.

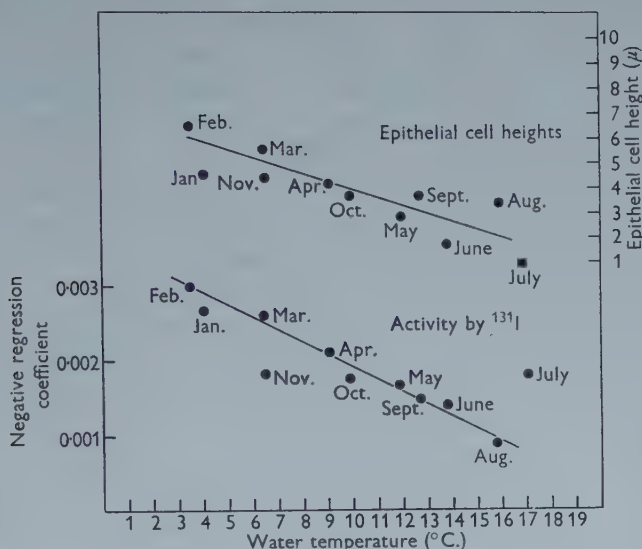


Text-fig. 2. Seasonal variation of the temperature of the water in which fish were living. Each point represents the mean of twice daily readings over the preceding period.

## DISCUSSION

In view of the recent detailed and excellent review of the literature on the teleost thyroid by Pickford (Pickford & Atz, 1957) and also that by Hoar (1957), it is not felt necessary to review here the many ideas on the function of the thyroid in teleosts. Periods of high activity have been indicated to occur at the same time as, among others, the following processes; larval morphogenesis, the metamorphosis of eels and flatfish, smoltification of salmon, and sexual maturation. After reviewing all the available data on the subject Pickford concludes that it appears increasingly probable in spite of evidences to the contrary, that the thyroid plays some role in the respiratory metabolism in teleosts. Huxley (1929) first suggested that the thyroid gland in poikilothermic animals may in some way act as a temperature-compensating mechanism, having a greater activity at lower environmental temperatures than at high ones. A comparison of the water temperature curve for 1956 and 1957 (shown combined in Text-fig. 2) and the thyroid activity curve (Text-figs. 1*a*, 1*b*) suggested that, with the exception of the June-July peak,

the activity of the gland was inversely proportional to the water temperature. This is clearly demonstrated in Text-fig. 3, where the glandular activity is shown plotted against the water temperature.



Text-fig. 3. The glandular activity measured by the epithelial cell height and radio-iodine plotted against the water temperature. The readings for glandular activity are taken from the trend curves drawn in Figs. 1*a* and *b*.

This relationship of glandular activity and water temperature is interpreted as further evidence that the basic function of the thyroid is concerned in the control of the animal's metabolism, in this case in such a fashion as to compensate for changes in the environmental temperature. Thus the release of thyrotropic hormone by the pituitary would seem to be influenced by the environmental temperature. Consideration of the summer activity peak suggests, however, that the release of thyrotrophic hormone is also affected by other stimuli—always, it would seem, when the response of the animal to these stimuli requires an increase in metabolism in whole or part. If this is indeed the case it would explain the various diverse occasions when an increase in thyroid activity has been indicated to occur. In this connexion it is perhaps illuminating to compare the results described in this paper with those previously reported (Swift, 1955). The activity of the gland of the maturing fish during the period of gonad maturation is much greater than that shown by the immature yearling fish at the same season, indicating an increase in thyroid activity at the time of gonad maturation. However, this does not account for the increase in activity in the yearling immature fish. It is interesting to note that it occurred around the time of the longest day, but until more is known about the effect of the gland on the metabolism, and also about the seasonal variation in glandular activity which presumably occurs in other species—in particular, species which mature at a different season than the brown trout—it is difficult to suggest a reason for this peak.

It is immediately obvious from Text-figs. 1*a*, *b* that an estimation of thyroid gland activity based solely on the histological criteria of epithelial cell height could be misleading. It is doubtful whether, in fact, the summer activity peak is demonstrated by this method, although in Text-fig. 1*a* a slight rise in epithelial cell height at this period could be indicated, but could also be the result of the curves for the 2 years being slightly out of phase at this point. However, when the thyroid sections are examined in the light of the iodine turnover rates additional information becomes apparent. The photomicrographs shown in Pl. 3, figs. 1–3, show the appearance of the gland at different times of the year. Pl. 3, fig. 1 is a section of a March gland and shows a typically spring active thyroid; by May the epithelium has become flatter and a great hyperplasia has taken place. Pl. 3, fig. 2 shows such a hyperplastic gland. In July, when the gland has again been shown to be active, the epithelium is still flat but the gland is highly vascular. Pl. 3, fig. 3 shows such a gland in which some of the follicles are ruptured and invaded by blood cells. This follicle rupturing has been noted before (Bargmann, 1939) and it has been suggested that this is one way in which the stored hormone is released into the blood stream.

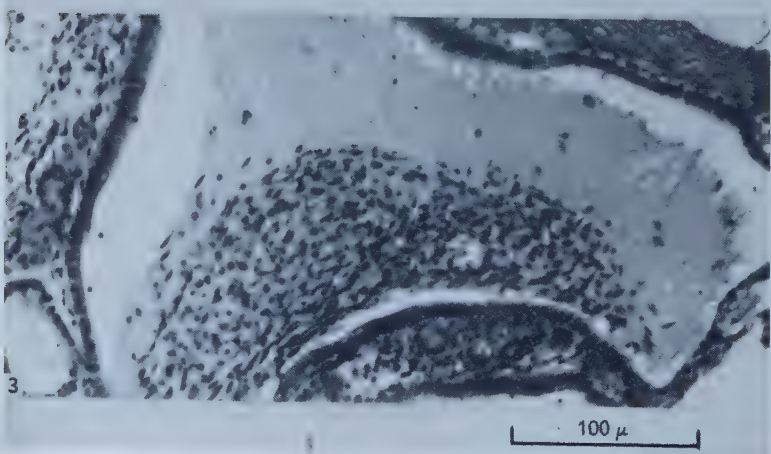
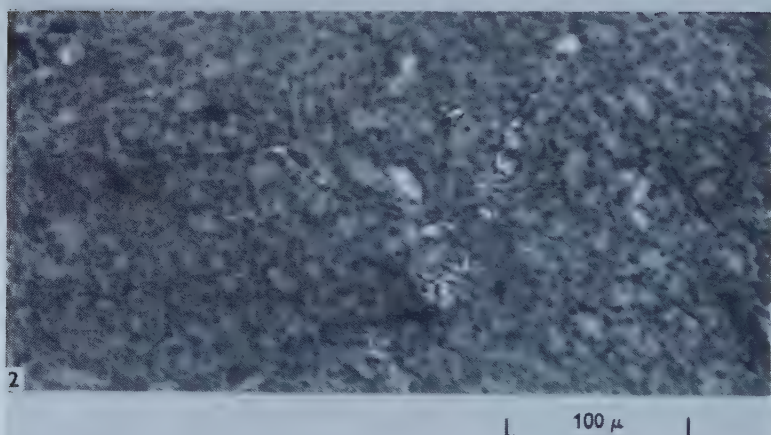
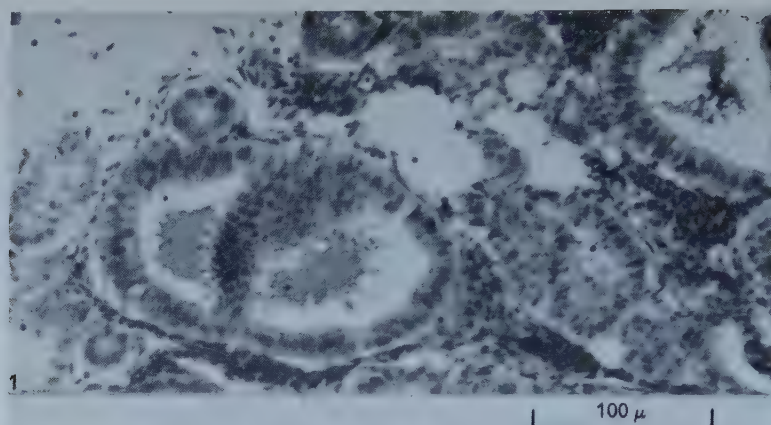
Consideration of these changes in the histological appearance, and the actual activity of the gland, has led to the suggestion that the rate of change of the level of thyrotropic hormone in the blood of the fish determines the mode of response of the thyroid to this hormone. Thus slow changes in the level, as it is supposed occur seasonally with changing water temperature, are responded to by a change in epithelial cell height, indicating a change in glandular activity. However, if the change in thyrotropic hormone level is rapid, as supposedly it is in July, then the stored hormone in the gland is released, the epithelial cells increasing in height later in August when their heightened activity replenishes the stores of hormone released in July. It is not suggested that this is a full account of the seasonal changes in behaviour of the thyroid—it does not account, for instance, for the hyperplasia found in May—but it is hoped that these ideas may stimulate further work on these lines especially with fish which spawn at times other than does the brown trout.

In conclusion it is perhaps worth noting that a study which would be very valuable—and which, as far as is known has never been made, presumably owing to the analytic difficulties involved—would be a survey of the iodine content of the water in which fish were living in conjunction with a survey of their thyroid activity. It has been shown that iodine deficiency will stimulate the thyroid and it may be there is a seasonal fluctuation of the iodine content in natural waters.

#### SUMMARY

1. Seasonal variations in the activity of the thyroid gland in yearling brown trout were measured by two methods; first, by the changes in thyroid epithelial cell height and, secondly, by the rate of loss of radio-iodine from the gland.
2. Peak thyroid activity was found to occur in spring with a second peak demonstrable by the radio-iodine technique in mid-summer.





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(Facing p. 125)

3. With the exception of this short burst of activity in July, the activity of the thyroid was found to be inversely proportional to the water temperature.
4. It is suggested that the thyroid is concerned in a temperature-compensating mechanism.
5. It is suggested that the rate of change of the thyrotropic hormone level in the blood determines the mode of response of the gland.

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#### EXPLANATION OF PLATE

- Fig. 1. Follicles from an active March gland.
- Fig. 2. An extreme example of a hyperplastic May gland.
- Fig. 3. A large follicle with low epithelium invaded by blood corpuscles, a July gland.

# THE ABSORPTION OF SODIUM IONS BY THE CRAYFISH, *ASTACUS PALLIPES* LEREBOULLET

## I. THE EFFECT OF EXTERNAL AND INTERNAL SODIUM CONCENTRATIONS

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(Received 25 August 1958)

### INTRODUCTION

The fact that many freshwater animals can absorb salts from the very low concentrations found in their environment was first demonstrated conclusively by Krogh (1937, 1938). He showed this by first reducing the salt content of the animal by washing, often for long periods, in continuously running distilled water. He was then able to demonstrate the uptake of sodium and chloride from dilute solutions and, in some cases, the concentration of these solutions was reduced below the limit of detection by his techniques. It was not possible to decide from his experiments whether ion absorption was a mechanism which came into action under conditions of salt depletion or whether it was a process which normally operated to maintain the salt balance of the animal.

The advent of the use of radioactive tracers provided another method for the measurement of ion uptake. The absorption of sodium ions has been demonstrated by this technique in the axolotl (Jørgensen, Levi & Ussing, 1946) and in the frog (Jørgensen, 1950; Jørgensen, Levi & Zerahn, 1954). More recently the long-lived sodium isotope,  $^{22}\text{Na}$  has been used to describe sodium uptake in the goldfish (Meyer, 1951; Sexton & Meyer, 1955) and in the crab, *Eriocheir sinensis* (Koch & Evans, 1956). In all these animals sodium ions are continually absorbed under normal conditions and the absorption is balanced by an equal loss of sodium from the excretory organs and through the integument.

Before completely accepting the results on tracer studies it is necessary to establish that the ion influx measured by the tracer technique is a true measure of the ion uptake rate. In the case of the isolated frog skin, which is the only material which has been thoroughly investigated from this point of view, the evidence strongly favours the identity of tracer influx and true uptake rate (see, for example, Ussing, 1954). Although this is encouraging, it cannot be argued, without further evidence, that it is necessarily true of the intact frog or of other animals. However, if tracer measurements are always accompanied by chemical analyses of the ion in question it is generally possible to assess the validity of the tracer measurements.

Although the uptake of ions has now been established in many aquatic animals, little is known of the factors which influence the rate of uptake. The extent to which

this is modified by environmental factors may be of great importance in the survival of the animal in any particular environment. On the other hand, there is evidence that the uptake rate may be regulated by the animal itself. Thus Jørgensen (1950) found that for both frogs and toads washing in distilled water for prolonged periods increased the sodium influx from 3 mM./l. NaCl solutions by a factor of 2 or more. Furthermore, Jørgensen, Levi & Zerahn (1954) found in the frog that sodium and chloride uptake mechanisms could be activated independently by sodium and chloride depletion, respectively.

The work described in this paper was designed to measure the normal uptake of sodium in the freshwater crayfish, *Astacus pallipes*, and to study the influence of one environmental factor—the external sodium concentration—on this. The quantitative effects of changes in the internal sodium content of the animal on the rate of uptake has also been studied in relation to the regulation of the uptake of sodium for the maintenance of sodium balance.

Some of these results have previously been briefly reported elsewhere (Shaw, 1958).

Previous knowledge of the absorption of ions by the crayfish is very limited. Krogh (1939, p. 91), quoting otherwise unpublished experiments, first showed the uptake of chloride by a single salt-depleted crayfish. He found that this animal absorbed chloride at a rate of 2.3  $\mu\text{M.}/40$  g. body weight/hr. and at a maximum rate of 6  $\mu\text{M.}/\text{hr.}$  Wikgren (1953) also reports the uptake of chloride in the crayfish, *Potamobius fluviatilis*, to be generally below 10  $\mu\text{M.}/100$  g./hr. although higher values were found. His results are based mainly on measurements of conductivity of the external solution, and the interpretation of these changes in terms of chloride movement requires great caution. The uptake of cations by the crayfish has not been previously reported.

#### MATERIAL AND METHODS

The crayfishes were obtained from the Freshwater Biological Association, Ambleside, and were kept in a running tap-water aquarium until used. For each experiment a single crayfish, weighing about 10 g., was transferred to a 1 l. Pyrex beaker containing the experimental solution, maintained at a constant temperature (12–13° C.). In all cases the experimental solutions consisted of de-ionized water or water containing pure sodium chloride. The effect of other ions on sodium uptake is reserved for consideration in another paper of this series. The volume of the experimental solution into which each crayfish was placed had to be carefully chosen. For animals weighing about 10 g. volumes of solution of the order of 50 ml. invariably resulted in low values for the uptake rate and this is probably due to the effects of accumulation of excretory products and the removal of oxygen from the water. On the other hand, if very large volumes were used, the accuracy of the sodium uptake measurements was considerably reduced. A compromise was adopted with a standard volume of 250 ml. for a crayfish of about 10 g.

Measurements of the sodium concentration of the external solution and of the blood were made by means of an EEL Flame Photometer.

Sodium influx was measured by using  $^{24}\text{Na}$  as a tracer. A crayfish was placed in 250 ml. of de-ionized water and  $^{24}\text{NaCl}$  was added to bring the total radioactivity of the solution to about  $3\ \mu\text{c}$ . Then sufficient non-radioactive  $\text{NaCl}$  was added to raise the total external sodium concentration to the desired level. A 1 ml. sample of the experimental solution was removed to a planchet, together with a drop of strong dextrose solution, and dried in an oven. The radioactivity of the sample was measured by means of an end-window Geiger counter in the usual manner. At approximately half-hourly periods during the experiment further samples were removed and measured in the same way. Generally, each experiment was continued for a total period of 4–6 hr. The radioactivity counting errors were less than  $\pm 2\%$  and the counting rate was corrected for the decay of the  $^{24}\text{Na}$  during the experiment. Because large differences in the concentration of sodium between the blood and the solution were always maintained, the build-up of radioactivity in the blood during the experimental period was never great enough for back-diffusion of radioactive ions to have a significant effect on the calculation of the sodium influx.

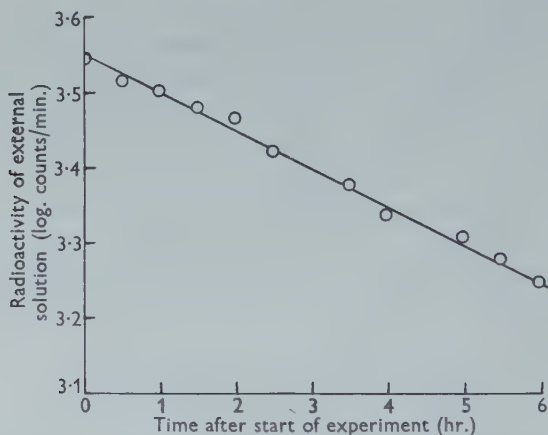


Fig. 1. The results of a typical influx measurement, showing the relation between the logarithm of the count rate in the external solution and the time after the start of the experiment.

In every experiment measurements of the total sodium concentration of the external solution were made at the beginning and at the end, and, in certain cases, during the course of the experiment. Generally, the conditions of the experiment were arranged so that only a small change in the external sodium concentration occurred.

To calculate the sodium influx, the logarithm of the measured radioactivities was plotted against the time at which the sample was taken. Under these experimental conditions this relationship was always satisfactorily linear as is shown in a typical example in Fig. 1. The sodium influx can be calculated from the slope of this line in the manner described by Jørgensen, Levi & Ussing (1946) in their studies on the uptake of sodium by the axolotl.

Thus if  $dy/dt$  is the rate of decrease of radioactivity,  $M$  is the influx,  $y$  is the relative radioactivity per unit volume of the solution and  $A$  is the total amount of sodium in the solution, then

$$dy/dt = -(M/A)y$$

if  $A$  is constant, then  $y = y_0 \exp -(M/A)t$ , where  $y_0$  is the initial activity. Hence  $\ln (y/y_0) = -(M/A)t$ . It may be noted that Jørgensen, Levi & Ussing also derived an expression for the influx under conditions where  $A$  was changing. However, their assumption that the influx was constant under these conditions is certainly not generally valid for the crayfish. It was found more satisfactory, therefore, to perform influx measurements under conditions where the external sodium concentration remained relatively constant.

In presenting the results of these experiments the following terminology will be used. *Sodium influx* ( $M$ ) refers to the influx of sodium ions as measured by the radioactive tracer technique. *Uptake rate* ( $U$ ) is the true influx of sodium ions. This cannot be measured directly and it may or may not be equal to  $M$ . *Net uptake* is the difference between the uptake rate and the loss rate and this can be measured by chemical analysis. *Loss rate* ( $L$ ) refers to the total loss of sodium ions, by outward diffusion, through the excretory organs and by any other means. The *equilibrium concentration* is the external sodium concentration at which a steady state is reached between the solution and the animal. The animal is in sodium balance and there is no net loss or gain of sodium.

In regard to the relation between the sodium influx and the uptake rate it is worth while making two observations at this point. First, the loss of radioactivity from the external solution can be accounted for approximately in terms of transfer of sodium ions across the integument to the blood: its disappearance cannot be ascribed to any adsorption phenomena. Measurements of the radioactivity of blood samples removed at the end of an experiment gave values consistent with the view that those radioactive ions which were lost from the external solution had been accumulated in the blood. These results are discussed in detail below. Secondly, although the very low external sodium concentrations used ensure that inward diffusion can play only a small part in the measured sodium influx, the possibility that exchange diffusion (Ussing, 1947) may account for a variable proportion of the sodium influx has always to be considered. However, the simultaneous measurement of sodium net uptake together with a knowledge of the loss rate make it possible to assess the importance of this type of interchange. This is also discussed below in the appropriate section.

## RESULTS

### (a) Sodium balance

The aquarium water contained 0.3 mM./l. Na. To measure the normal sodium influx, animals were transferred from the aquarium to experimental solutions containing this concentration of sodium chloride. The results of these measurements are shown in Table 1. The mean value is 1.5  $\mu\text{M.}/10\text{ g./hr.}$

Table 1. *Sodium influxes from 0.3 mM./l. NaCl for animals in balance at this concentration*

Specimen no.	Body weight (g.)	Sodium influx ( $\mu\text{M./hr.}$ )	Specific sodium influx ( $\mu\text{M./10 g./hr.}$ )
I	12.9	1.8	1.4
II	12.0	1.8	1.5
14	7.2	0.8	1.1
15	14.0	2.8	1.8
17	12.0	2.0	1.7
			Mean = 1.5

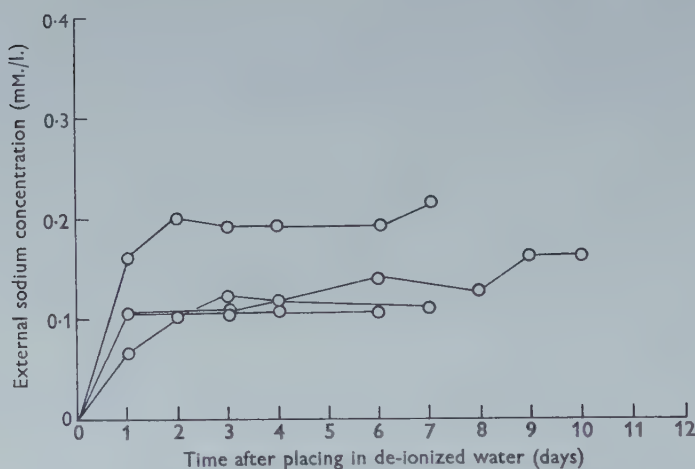


Fig. 2. The attainment of an external equilibrium concentration by four crayfish each placed in 250 ml. of de-ionized water.

If the sodium influx approximates to the uptake rate and if the animal is in sodium balance, then the sodium influx should be matched by an equal rate of sodium loss. The loss rate was estimated in the following way. When a crayfish was placed in 250 ml. of de-ionized water with no added sodium chloride, sodium was initially lost to the water. The concentration of sodium in the water gradually increased until it reached a level at which it remained more or less constant for several days. The results of several experiments of this kind are shown in Fig. 2. The increase in sodium concentration of the water over the first few hours was used to calculate the rate of loss of sodium from the animal. Table 2 shows loss rates calculated in this way for a number of different individuals. The mean value for the loss rate ( $1.5 \mu\text{M./10 g./hr.}$ ) is the same as that of the influx and, where the same individuals have been used for both measurements, there is generally fairly good agreement for the two values. This estimate of the loss rate should not be accepted without qualification. It is only strictly valid for loss into de-ionized water—it does not necessarily follow that the loss rate will be the same at all external concentrations or in all

physiological states of the animal. This question is discussed again in more detail below.

It is interesting to see where the main loss of sodium occurs. Krogh (1939) calculated that  $0.17 \mu\text{M./10 g./hr.}$  of chloride was lost through the excretory organ. If the sodium loss is of the same order, then it is clear that only about one-tenth of the sodium could come out by this means. The remaining 90% presumably diffuses out through the integument, probably largely through the gills. It is not certain if the same is true for the behaviour of the chloride ion, since the situation for this ion is far from clear. In a single experiment Krogh found the rate of loss of chloride to distilled water was not much greater than the expected loss through the excretory organ. On the other hand, Wikgren (1953) states that the ratio of the loss of chloride through the body surface to that through the urine is 10-20:1, but the data from which he calculated this ratio is by no means clearly presented.

Table 2. *Rates of loss of sodium into de-ionized water and the minimum equilibrium concentrations*

Specimen no.	Rate of sodium loss. $\mu\text{M./10 g./hr.}$	Minimum equilibrium concentration (mm./l.)
1	1.5	0.02
8	1.4	—
10	—	0.03
11	—	0.04
13	—	0.03
14	1.1	0.03
15	1.4	—
17	2.2	0.09
	Mean = 1.5	Mean = 0.04

Returning now to Fig. 2, it is clear that the equilibrium concentration for those animals transferred from the aquarium to de-ionized water is not necessarily the same as the sodium concentration of the aquarium water. Indeed, it is almost always rather lower. Fig. 2 shows a number of individuals which have come into sodium balance with an external sodium concentration of between 0.1 and 0.2 mm./l. Furthermore, if animals which have already come into balance after placing in de-ionized water are transferred to a new volume of de-ionized water, then the new final equilibrium concentration will be lower than before. Progressive loss of sodium gradually reduces the equilibrium concentration further until, finally, a minimum equilibrium concentration, or threshold, is reached. Further removal of sodium cannot reduce the equilibrium concentration below this minimum value. In Table 2 the minimum equilibrium concentrations for a number of individuals are listed. The variability is fairly large, but the values generally lie below 0.1 mm./l. and the mean value is 0.04 mm./l.

It is noteworthy that the minimum equilibrium concentration on no occasion approached zero, as recorded by Krogh after the absorption of chloride from a dilute Ringer solution by a salt-depleted crayfish. This reason for this difference

is not clear. Although it is possible that lower minimum sodium equilibrium concentrations could result if larger volumes of de-ionized water were used, this is not the explanation of the divergence from Krogh's result. It can be calculated from his figures that he must have used only 70 ml. of the Ringer solution for a 40 g. crayfish. It may well be that there is a real difference in the behaviour of the mechanisms controlling the uptake of sodium and chloride respectively, but other possibilities, such as species differences, cannot be ruled out.

The attainment of sodium balance at low external concentrations after salt depletion is probably a feature common to many freshwater animals, although the value of the minimum concentration is variable. Thus in the freshwater crab, *Eriocheir sinensis*, Koch & Evans (1956) found that the minimum equilibrium concentration could not be reduced below between 0.2–0.5 mM./l. On the other hand, the East African freshwater crab, *Potamon niloticus* (Shaw, 1959), closely resembles the crayfish in this respect, attaining balance at a minimum concentration of about 0.05 mM./l.

The facts that the crayfish comes into balance with a certain external sodium concentration, and that this concentration varies according to the sodium content of the animal, make it clear that both the external sodium concentration and the internal sodium content must exert some influence over either the rate of sodium uptake or the rate of loss, or over both. The effect of these two factors on the sodium uptake rate will now be considered.

(b) *The effect of the external sodium concentration on the sodium influx*

The sodium influx at different external sodium concentrations was measured by the method described above, although, since it was necessary to make several measurements on a single individual, a number of precautions had to be taken. In the first place, to avoid back-diffusion of radioactive ions, a period of time (generally 1 day) was allowed to elapse between each influx measurement. During this time the radioactivity of the blood had decayed to a suitable level. This meant that only a few influx measurements on each individual could be made in 1 week and the complete experiment had to be extended over several weeks. In the second place, during an influx measurement at a high external concentration there was a considerable net uptake of sodium: this increased the internal sodium content and this, in its turn, reduced the influx (see below). To get over this difficulty the following experimental procedure was adopted. The animal was first subjected to sodium loss by treatment in de-ionized water until the animal was in balance with its minimum equilibrium concentration. Further sodium was then removed and in this situation it was found that the influx was not affected by small changes in the internal sodium content. As an additional precaution, at the end of each influx measurement at a high external concentration the animal was placed in de-ionized water to remove the sodium which had been absorbed. Care was also taken that the influx measurements were not made with progressively increasing or decreasing external concentrations; the external concentrations were selected at random.

The adoption of the above procedure made it possible to obtain consistent and reproducible measurements of sodium influx over a wide range of external concentrations. The results obtained for a number of individual animals are shown in Figs. 3 and 4. It might be supposed that as the external concentration is increased, the number of sodium ions making contact with the sodium-transporting system will also be increased and the sodium influx will increase in proportion. If such were the case, there would be a linear relation between the external concentration and the sodium influx. Such a relation clearly does not exist except perhaps at very low external concentrations: in all cases the influx levels off to approach a maximum rate at an external concentration of about 1 mM./l.

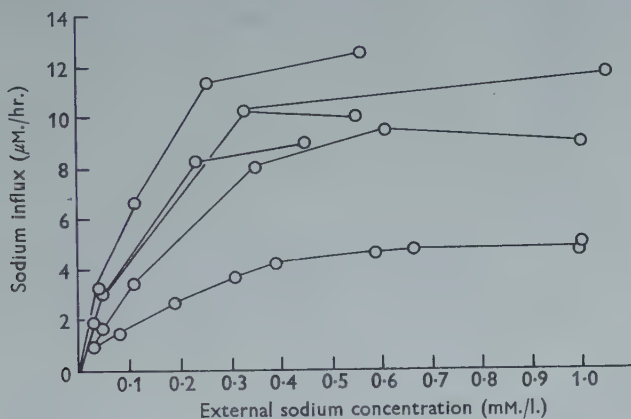


Fig. 3. The relation between the sodium influx and the external sodium concentration in several individuals.

This phenomenon has not been previously observed in any intact animal, but it does have a parallel in the transport of sodium by the isolated frog skin. Ussing (1949) first observed that the relation between the sodium influx and the external sodium concentration was non-linear, although he found no maximum even with external concentrations of the order of 100 mM./l. The question was later re-investigated by Kirschner (1955) who, calculating the influx by the difference between the outflux and the short-circuit current, found a definite maximum at an external concentration of about 35 mM./l. At a higher external concentration the influx appeared to decline. Kirschner sought to explain the non-linear relation in terms of the mediation of a sodium carrier which formed an unionized complex with sodium ions and which became saturated at high external sodium concentrations. On this assumption he devised an equation relating the influx to the external concentration, but this was not completely adequate to account for his experimental results. Both Ussing and Kirschner used external concentrations which were much in excess of the normal physiological range.

In the crayfish the non-linear relation between influx and concentration becomes apparent at concentrations well below that found for the frog skin. The maximum

influx is approached at a concentration within the normal physiological range. Without making any assumptions of the presence of sodium carrier complexes, it seems probable that in the crayfish sodium ions are transported inwards by a mechanism which is limited in its rate of operation and therefore becomes saturated at higher external concentrations. This type of relation recalls the relationship between the rate at which an enzyme breaks down its substrate and the concentration of the substrate—a situation which has been successfully described by the well-known Michaelis equation. It is interesting to see if this equation is applicable in the case of the sodium influx. Fig. 4 shows the relation between the sodium influx

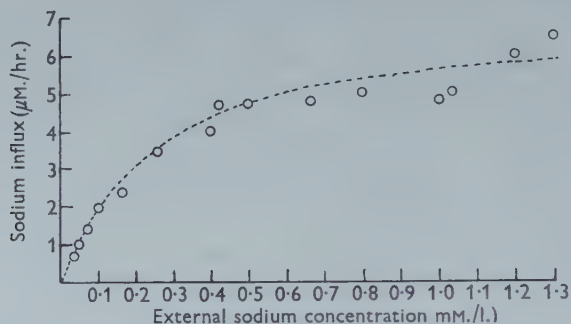


Fig. 4. The relation between the sodium influx and the external concentration in a single specimen (crayfish no. 14).

and the external concentration in one animal on which a large number of influx measurements had been made. On the same figure the dotted line represents the expression,  $M = Kc/(K_m + c)$ , where  $M$  is the influx,  $c$  the external sodium concentration, and  $K$  and  $K_m$  are constants with arbitrarily chosen values of 7 and 0.25, respectively. This expression clearly provides a reasonable approximation of the relation between  $M$  and  $c$  over the range which has been studied. However, there may well be other expressions which would fit equally well, or even better, and no particular significance is attached to this one. It does seem likely, though, that the true relation would (like the Michaelis equation) contain a factor determining the maximum influx and another, the rate at which the maximum was reached. On these grounds it may be suggested that sodium ions are transported inwardly by a saturable and rate-limited system which is characterized by a very high affinity for sodium ions.

Before proceeding with the next stage of the analysis, we must now examine the question of the equality between the sodium influx, as measured by the tracer technique, and the true uptake of sodium against the concentration gradient. This has been investigated by comparing the measured influx with the net uptake (or loss) which occurred during the experiment. If the influx correctly measures the total sodium uptake, then the difference between the influx and the net uptake must equal the rate of loss. The results of these experiments on one animal are shown in Fig. 5 and further examples are shown in Table 3 (specimens 10, 11 and 1).

We can first consider in detail the results which were obtained on crayfish no. 14 (body weight 7.2 g.), which are those shown in Fig. 5. The conclusions drawn concerning the behaviour of this animal are valid also for those animals listed in Table 3. Over the lower part of external concentration range the value of the influx minus the net uptake is about  $0.8 \mu\text{M./hr.}$  (this corresponds to a value of  $1.1 \mu\text{M./hr./10 g.}$  body weight), and this agrees well with the rate of loss as measured by the loss of sodium into de-ionized water (see Table 2, specimen no. 14). However, as the external concentration increases, the value of the influx minus the net uptake also increases to a value of about  $1.8 \mu\text{M./hr.}$  Now these results could obviously be explained on the grounds that the loss rate was not constant but increased at the higher external concentrations. Fortunately, this hypothesis can be tested in the following way: if the influx at the high external concentrations is reduced to a value

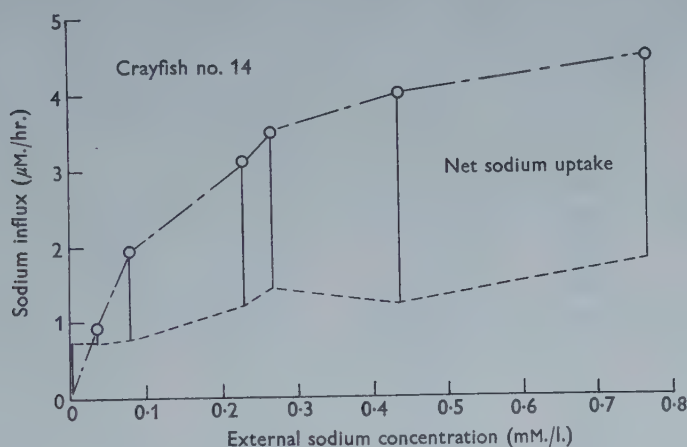


Fig. 5. The relation between the sodium influx and the external sodium concentration, together with measurements of the net sodium uptake (indicated by the vertical lines). Upper curve — — — is sodium influx; lower curve — — — is sodium influx minus net uptake.

below about  $1.8 \mu\text{M./hr.}$  then a net loss of sodium should occur. A reduction in the influx was brought about by two different methods—(1) by the increase in the internal sodium content of the animal and the consequent adaptation to a higher equilibrium concentration (the effect of the internal sodium content in reducing the influx is discussed in detail in the following section; here it is used simply to lower the influx at the high concentrations), (2) by the use of an influx inhibitor in the external solution (in fact, a mixture of 5%  $\text{CO}_2$  in air was bubbled through the solution). The application of these two methods to crayfish no. 14 are shown in the lower half of Table 3 (Exps. 14b and d-g). At the higher external concentrations (for example,  $0.31 \text{ mM./l.}$  in Exp. 14d and  $0.445 \text{ mM./l.}$  in Exp. 14g), despite the lowered influx (compared with that shown in Fig. 5 for these concentrations), a net uptake of sodium still occurred. The value of the influx minus the net uptake ( $0.53$  and  $1.0 \mu\text{M./hr.}$  for 14d and 14g, respectively) had fallen from the previous values (shown in Fig. 5) and now approximated to the normal loss rate ( $0.8 \mu\text{M./hr.}$ , Fig. 5).

Now it might be argued that the reduction in the influx, although effected by two different methods (i.e. by the use of  $\text{CO}_2$  as in 14*d*, or by the increase in internal sodium content as in 14*g*), was always accompanied by a decrease in the true loss rate as well. If this was so, then one would expect that at the lower external concentrations (for example, Exps. 14*b* and 14*f* in Table 3) the value of the influx minus the net uptake (or loss) would be less than the normal loss rate. In fact these values are not significantly different from the normal loss rate (14*b*, *f*, 0.7 and 1.0  $\mu\text{M./hr.}$  respectively, compared with the normal loss rate of 0.8  $\mu\text{M./hr.}$ ). On these grounds it seems reasonable to suppose that the true rate of loss is practically constant over the range of external concentrations studied.

Table 3. *A comparison between the sodium influx and the net sodium uptake in animals adapted to low- and high-equilibrium concentrations and in the presence of 5%  $\text{CO}_2$*

Specimen no.	Equilibrium concentration (mM./l.)	External concentration (mM./l.)	Sodium influx ( $\mu\text{M./hr.}$ )	Net uptake ( $\mu\text{M./hr.}$ )	Influx minus net uptake
10	0.03	0.11	3.4	1.7	1.7
	0.03	0.55	10	5.3	4.7
11	0.04	0.04	1.5	-0.2	1.7
—	0.04	0.56	10	5.2	4.8
1	0.03	0.26	4.4	2.5	1.9
—	0.03	0.45	5.4	2.5	2.9
14 <i>a</i>	0.03	0.08	1	—	—
<i>b</i>	0.03	0.08	0.25	-0.45	0.7
		+ 5% $\text{CO}_2$			
<i>c</i>	0.03	0.31	3.5	2.0	1.5
<i>d</i>	0.03	0.31	1.2	0.67	0.53
		+ 5% $\text{CO}_2$			
<i>e</i>	0.25	0.25	1.1	-0.2	1.3
<i>f</i>	0.25	0.24	1.0	0.0	1.0
<i>g</i>	0.25	0.445	1.8	0.8	1.0

It must therefore follow that whereas at the lower range of external concentrations the influx is a reasonable measure of the true uptake rate, at the higher concentrations the influx is distinctly greater. In the case of crayfish no. 14 (Fig. 5) at an external sodium concentration of 0.77 mM./l. the sodium influx is 4.5  $\mu\text{M./hr.}$ , whereas the uptake rate is only 3.5  $\mu\text{M./hr.}$  (i.e. the net uptake, 2.7  $\mu\text{M./hr.}$  + loss rate, 0.8  $\mu\text{M./hr.}$ )—some 22% lower. This difference can probably be explained in terms of the type of interchange between radioactive and non-radioactive ions which Ussing (1947) has called 'exchange diffusion'. It is significant that the divergence between the influx and the uptake rate becomes apparent only when the transporting system approaches saturation. It is probably analogous to a leaky pump which works efficiently at low rates of delivery but develops a considerable leak-back when overworked.

An increase in the sodium outflux (measured by a tracer technique) with increasing external sodium concentration has been observed in the isolated frog skin by Ussing (1949) and Kirschner (1955). Kirschner attempted to explain this phenomenon

on the grounds that the outwardly diffusing sodium ions interacted with a hypothetical sodium carrier system and that some of them were carried back again. If the rate of backward movement is proportional to the concentration of free carrier present, then it will be smaller at the higher external concentrations, and hence the outflux will be greater. This hypothesis is not applicable to the loss of sodium by the crayfish. If it was, then a reduction in the influx from low external concentrations should result in a considerable increase in the rate of loss of sodium and this is not found (see Table 3, 14*b, f*).

(c) *The effect of the internal sodium concentration on the sodium influx*

When sodium is lost from an animal by treatment in de-ionized water the consequent decrease in the internal sodium content has a pronounced effect on the sodium influx. Table 4 shows the influxes from 0.3 mM./l. NaCl measured in animals taken from the aquarium and also after they have lost sufficient internal sodium to reduce their external equilibrium concentrations to the minimum level. The influx was increased from three to five times.

Table 4. *Sodium influx in animals adapted to an external equilibrium concentration of 0.3 mM./l. Na and also adapted to their minimum equilibrium concentration*

Specimen no.	Normal sodium influx from 0.3 mM./l. NaCl ( $\mu\text{M.}/\text{hr.}$ )	Maximum sodium influx from 0.3 mM./l. NaCl ( $\mu\text{M.}/\text{hr.}$ )	Ratio of maximum to normal influx
I	1.8	9.4	5.2
II	1.8	9.4	5.2
13	1.0	3.6	3.6
14	0.8	3.8	4.8
15	2.8	7.4	2.6
17	2.0	6.8	3.4

These observations reveal little about the quantitative aspect of the effect of the internal sodium content. This was analysed by measuring the influx when known amounts of sodium had been introduced or removed from the animal. Artificial methods of changing the sodium content, such as by the injection of sodium chloride solutions, were avoided and changes were induced by physiological means. Thus, to increase the sodium content, the animal was allowed to absorb a measured amount of sodium from an external solution more concentrated than the equilibrium concentration to which the animal was adapted; and conversely, to decrease the sodium content, the animal was placed in de-ionized water until the required amount of sodium had leaked out. The method may be illustrated by reference to a typical experiment. An animal was first adapted to an external equilibrium concentration of, say, 0.3 mM./l. NaCl. The influx was then measured at a known external sodium concentration. The animal was then placed in a known volume of de-ionized water and allowed to lose a measured amount of sodium (calculated from the increase in sodium concentration of the external solution). The influx was now measured again

at the same external sodium concentration as before. The animal was again placed in de-ionized water and a further amount of sodium removed. The influx was measured once more. The removal of sodium and the measurement of the new influx was repeated several more times. Between each new influx measurement 24 hr. was allowed to elapse to ensure that the new influx rate was fully established.

In an alternative form of the experiment the animal was first adapted to a very low equilibrium concentration. The influx was measured as before and then the animal was allowed actively to absorb a measured amount of sodium. The new influx was again recorded and then the procedure repeated several times more.

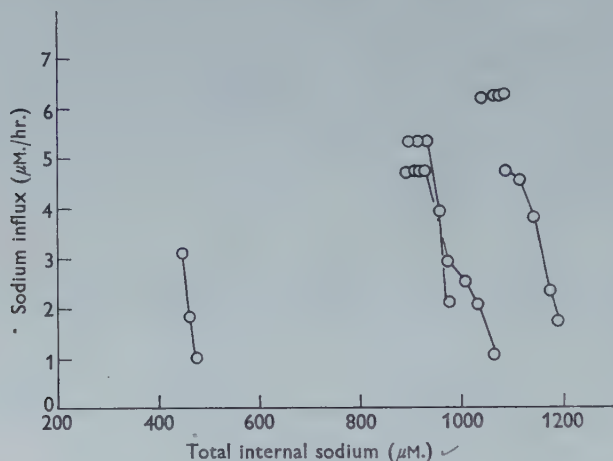


Fig. 6. The relation between the sodium influx and the total freely exchangeable internal sodium. Points joined together represent measurements on one individual.

The results of these experiments are shown in Fig. 6. It can be seen that a small decrease in the internal sodium content causes a steep rise in the influx until a maximum is reached: after that further sodium loss has no effect on the influx. A loss of only 10  $\mu\text{M}$ . Na often affected the influx, whereas the loss of 100  $\mu\text{M}$ . or less was generally sufficient to change the influx from the normal to the maximum level.

We must now establish what these amounts represent in terms of the total sodium content of the animal, or, rather, the total sodium which is free to exchange rapidly with the external sodium. This can be calculated from the volume of fluid in the animal which contains this sodium (the 'sodium space') and its sodium concentration. The 'sodium space' consists, of course, largely of blood, but the two volumes may not necessarily be identical, since some tissues may also contain freely exchangeable sodium. The 'sodium space' was calculated from tracer experiments. An animal was placed in a known volume of solution containing some  $^{24}\text{Na}$  and some of this was absorbed into the blood. The radioactivity which was lost from the outer solution was measured, after correction for decay, and compared with the specific activity of a blood sample. From this the volume of fluid into which the radioactive ions must have been transferred and dispersed was calculated. The

blood sodium concentration was also measured and it was assumed that the sodium concentration was uniform throughout the whole of the 'sodium space'. From these two measurements the total freely exchangeable sodium content of the animal was calculated.

Table 5. *Estimations of the total freely exchangeable sodium*

Specimen no.	Body weight (g.)	Estimated sodium space (ml.)	% of body weight (v/w)	Blood Na concentration (mm./l.)	Total sodium ( $\mu$ M.)
16	9.8	3.05	31.1	—	—
14	7.2	2.7	37.5	193	521
19	14.6	7.2	49.3	152	1094
20	14.8	5.2	35.1	166	863
21	13.0	5.6	43.1	166	930

The results of measurements of this kind on several individuals are given in Table 5. The measurements of blood sodium concentration agree with those reported in the literature previously (see, for example, Bogucki, 1934). The sodium space (mean value = 37.2% of body weight) is probably not much larger than the true blood volume. No figures for this appear to be available for *Astacus*, but Prosser & Weinstein (1950) recorded the blood volume of the crayfish, *Cambarus virilis* as 25.1% of the body weight. However, the body weights range from 10 to 48 g. and if only the smaller specimens are considered (16 g. or less) a much higher value of 34.5% is obtained. A value similar to this has also been found for the blood volume of *Carcinus maenas* (Webb, 1940) and for *Eriocheir sinensis* (Krogh, 1939).

With a knowledge of the total internal sodium content, we may now refer back to Fig. 6, where this is also recorded. It is now possible to see that changes in the influx can be induced by sodium losses which represent only 1% of the total sodium content. Further, the influx may be changed from the normal to the maximum value by the removal of only 5–10% of the internal sodium.

The next question that arises is whether the changes in internal sodium content correspond to changes in the internal sodium concentration (i.e. whether the 'sodium space' remains constant). This was tested by first measuring the blood sodium concentration of an animal with a normal influx and in balance with an external concentration of 0.3 mm./l. NaCl, and then measuring the concentration again after sodium had been removed from the animal and the maximum influx attained. These results are shown in Table 6. In all cases a measurement of the blood concentration at maximum influx was obtained before and after the measurement at normal influx. The blood concentration at normal influx is always higher than at maximum influx. Furthermore, the difference between the concentrations is of the same order (i.e. about 10%) as the percentage change in sodium content required to alter the influx from one extreme to the other. It is, therefore, almost certain that it is the internal sodium concentration which is the factor responsible for determining the influx.

Table 6. *Blood sodium concentrations in animals adapted to 0.3 mM./l. NaCl and also adapted to their minimum equilibrium concentrations (maximum influx level)*

Specimen no.	Blood concentration at maximum influx level (mM./l.)	Blood concentration at normal influx level (mM./l.)	Blood concentration at maximum influx level again (mM./l.)
19	152	195	170
20	166	186	173
21	166	177	170
Means	161	186	171

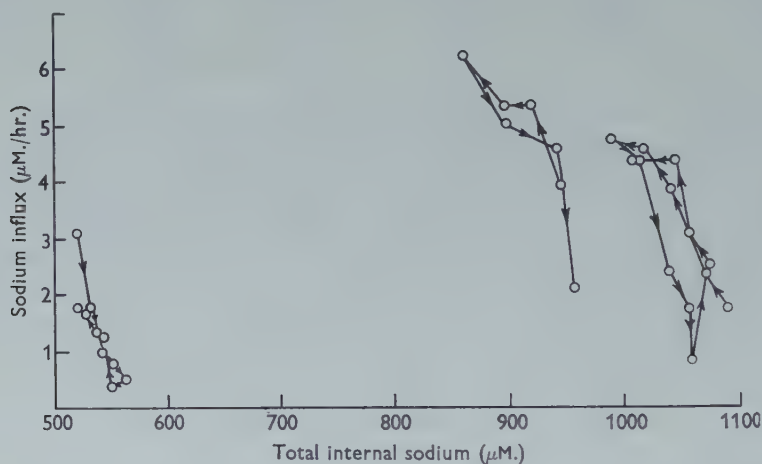


Fig. 7. The relation between the sodium influx and the total freely exchangeable internal sodium in three individuals. The arrows represent the direction in which the sodium changes have occurred.

The effect of the *external* sodium concentration on the influx is characterized by its immediate and reproducible action, but the responses to changes in the *internal* sodium concentration appear to be of a somewhat different nature.

In the first place, the removal of sodium from, or its addition to, the animal does not have a completely reversible and reproducible effect on the influx. Fig. 7 shows, in several individuals, the internal sodium changes inducing successive increases and decreases in the influx. It will be seen that there is not complete coincidence of the influxes at any given sodium level and that hysteresis is often in evidence. It is possible that it is not the absolute internal sodium concentration which determines the influx but the change from one concentration to another. The rate at which this occurs may be of some importance.

In the second place, the change from one level of influx to another, induced by a change in the internal sodium content, is rather slow. An estimate of the time for the adaptation to a new rate was made in the following way: an animal with the influx at the maximum level was placed in a solution of higher external concentration (about 0.6 mM./l. NaCl) containing some  $^{24}\text{Na}$ . A continuous record of the fall

of activity and the change in sodium concentration of the external solution was made. Net uptake of sodium occurred and this increased the internal sodium content. The experiment was continued over a period of 26 hr. and the time taken for the increase in the sodium content to affect the influx was noted. The results obtained for two individuals are shown in Fig. 8. Here the actual records are shown and the influx is indicated by the slope of the upper curves representing the decrease in radioactivity of the external solution. After the first 6 hr. little change in the influx had occurred, although a considerable amount of sodium had been absorbed. In crayfish no. 10, from 6 to 14 hr. a gradual change in the influx took place and at the end of this time the final new rate was established and the external concentration had fallen to the new equilibrium level. In crayfish no. 11, the influx remained unaltered for a longer period and then changed over to the new rate between 9 and 15 hr. after the start.

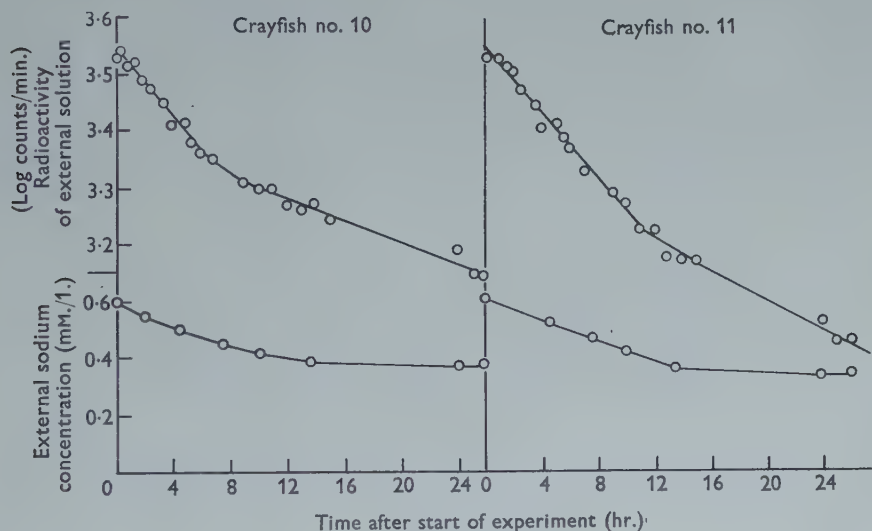


Fig. 8. Experimental results showing the time taken for the influx level to alter as a result of sodium absorption. The influx is calculated from the slope of the upper curves.

(d) *The interdependence of external and internal sodium concentrations*

Since both the internal and external sodium concentrations affect the sodium influx, sodium balance must be achieved at an external concentration, where the combined effect of these two factors produces sodium influx which just equals the loss rate. Changes in the internal sodium concentration must, therefore, affect the external equilibrium concentration. Table 7 illustrates, quantitatively, the effects of the removal or addition of sodium on the external equilibrium concentration. As would now be expected, the losses or gains of sodium required to change the equilibrium concentration from a low level to a normal level (and, in the case of specimen no. 1, back again) are of the same order as those which produce a change in the influx from the normal to the maximum level.

Table 7. *The effect of changes in the internal sodium content on the external equilibrium concentration*

Specimen no.	Initial equilibrium concentration (mm./l.)	Net gain of sodium ( $\mu$ M.)	Final equilibrium concentration (mm./l.)
I	0.07	+60	0.375
I	0.375	-51	0.04
I	0.04	+71	0.36
4	0.04	+58	0.384
10	0.04	+56	0.366
11	0.06	+68	0.336

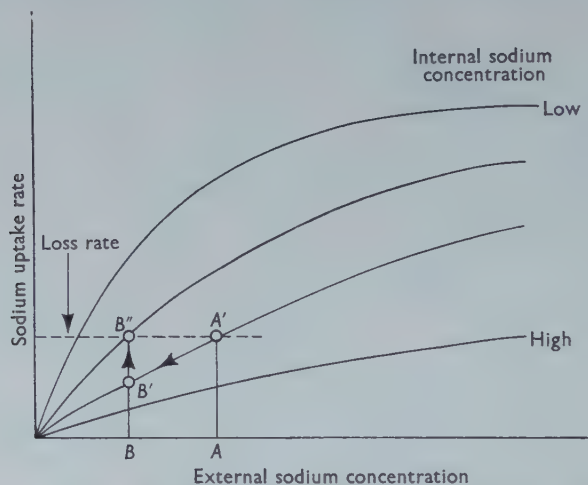


Fig. 9. A diagram representing the relationship between the three variables, the sodium uptake rate, the external sodium concentration and the internal sodium concentration.

## DISCUSSION

In the analysis described above the behaviour of the crayfish in pure sodium chloride solutions only has been considered—the effects of other ions will be analysed later. With this limitation, it is possible to build up a picture of sodium balance in the crayfish, within its normal physiological range of sodium concentrations. In the system under consideration there are only three important variables; (a) the sodium uptake rate, (b) the external sodium concentration, and (c) the internal sodium concentration. The rate of loss, for reasons given above, can probably be considered as a constant. The three variables may be related to each other in the form of a family of characteristic curves, such as is shown diagrammatically in Fig. 9. This, of course, is an oversimplification, since the precise nature of the curves has not yet been fully determined, but they serve for a qualitative demonstration of the balance conditions. The effect of a change in one of the variables can be followed. Let  $A'$  represent the uptake rate ( $U$ ) at a certain external sodium concentration ( $A$ )

and let the animal be in sodium balance (i.e.  $A' =$  the loss rate,  $L$ ). If the external concentration should now fall to  $B$ , then the uptake rate falls along the curve to  $B'$ . Now  $U$  is less than  $L$ , sodium is lost from the animal and the internal sodium concentration is decreased. The uptake rate is now increased to  $B''$  on the curve which corresponds to the new internal sodium concentration and balance is re-established at the new external sodium concentration. The system is clearly a self-balancing one. Because a small change in the internal sodium concentration may bring about a large change in the uptake rate, a tenfold fall in the external sodium concentration may be compensated by a decrease in the internal sodium of less than 10%.

There seems little reason to doubt that the effect of the external sodium concentration on the sodium transporting system is a direct one, exerting its action by increasing or decreasing the number of sodium ions which come in contact with the transport sites. The effect of the internal sodium concentration is more difficult to explain. The fact that its action may be delayed and is also somewhat unpredictable suggests that it may not affect the transporting system directly. It is possible that changes in the blood sodium concentration may produce an indirect effect. It might, for example, stimulate the liberation of a hormone, rather in the same way as, in the mammal, changes in the blood osmotic pressure provoke the liberation of the anti-diuretic hormone which, in its turn, regulates the reabsorption of water by the kidney tubules (Verney, 1947).

#### SUMMARY

1. The effects of external and internal sodium concentrations on the uptake of sodium ions by the crayfish, *Astacus pallipes*, has been studied.

2. The normal sodium influx, measured with  $^{24}\text{Na}$ , from 0.3 mM./l. NaCl solution is 1.5  $\mu\text{M.}/10$  g. body weight/hr. The rate of loss of sodium to de-ionized water has roughly the same value.

3. Net loss of sodium reduces the external sodium concentration required for sodium balance. The minimum equilibrium concentration is about 0.04 mM./l. NaCl.

4. The relation between the external sodium concentration and the sodium influx is non-linear. The influx has a maximum of about 10  $\mu\text{M.}/10$  g./hr. at an external concentration of approx. 1 mM./l.

5. The  $^{24}\text{Na}$  influx is a true measure of the sodium uptake rate at low external concentrations. At higher concentrations the influx may exceed the uptake rate by some 20%.

6. Net loss of sodium increases the influx by three to five times. Loss of 5–10% of the total internal sodium increases the influx from the normal to the maximum level. A 1% change has a significant effect on the influx. Changes in the internal sodium content reflect changes of the blood sodium concentration.

7. A scheme is suggested whereby the external and internal sodium concentrations interact together on the influx to produce a self-regulating system which maintains the animal in sodium balance.

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# SOLUTE AND WATER BALANCE IN THE MUSCLE FIBRES OF THE EAST AFRICAN FRESH-WATER CRAB, *POTAMON NILOTICUS* (M. EDW.)

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## INTRODUCTION

One of the important factors in the penetration of marine animals into brackish water and eventually into fresh water has been the ability of the cells of the organism to withstand and adapt themselves to a very great reduction in the total concentration of the blood. There are obviously many ways in which this might be accomplished. In the case of the muscle fibres of the marine and estuarine crab, *Carcinus maenas*, cell adaptation involves the reduction of the osmotic activity of the cell contents by the removal of certain small nitrogenous compounds, such as free amino acids (Shaw, 1958*a*).

It is possible that the ability of cells actively to regulate their composition in response to changes in blood concentration, and hence preserve the normal water balance of the cell, is a special characteristic of brackish water animals. These animals normally withstand large changes in the composition of their environment, and it is of great interest to know whether these abilities are still possessed by fresh-water animals, which have been derived from marine forms but are now living in a much more stable environment.

In the case of the crab, *Eriocheir sinensis*, which spends much of its life in fresh water but returns to the sea to breed, the muscles from the marine animals contain greater amounts of free amino acids than those in animals from fresh water (Duchâteau & Florkin, 1955), although it is not known to what extent these changes are due to changes in water content.

It has been suggested (Ramsay, 1954) that one of the reasons why many fresh-water animals are unable to survive in concentrations of sea water much above that of the normal blood concentration, is due to the fact that the muscles are unable to increase their amino acid content.

The East African fresh-water crab, *Potamon niloticus*, is a very favourable animal on which to pursue this question further. In the first place, it is restricted to fresh water and shows the characteristic inability of fresh-water animals to survive in sea water (Shaw, 1959). In the second place, it is reasonably closely related to *Carcinus* and therefore the behaviour of its muscle fibres may be compared with that of *Carcinus* muscle fibres under similar circumstances.

## MATERIALS AND METHODS

The crabs were collected from the shores of Lake Victoria, at Entebbe and maintained in a laboratory aquarium with circulating lake water. For experiments on the effect of different concentrations of sea water on the animals, specimens were kept, singly, in jars each containing about 500 ml. of the appropriate solution, which was changed at frequent intervals. The crabs remained in the experimental solutions for a week or longer, except in the case of those animals in undiluted sea water. In this solution the animals died within a week and so they were removed from the solution for analysis after only 3 days. It cannot be certain, therefore, that in these animals steady-state conditions were established fully at the time of the analysis. Since natural sea water was not available, all the experimental solutions consisted of artificial sea-water solutions made up from a mixture of standard salt solutions as described below. None of the solutions contained sulphate. In some experiments magnesium-free solutions were used without any noticeably different effects on the animals or on their muscles. The effects described, therefore, cannot be attributed to increased concentrations of magnesium or sulphate ions in the blood.

The procedure for the muscle analysis followed that used for similar analyses on *Carcinus* muscle (Shaw, 1955*a*, *b*; 1958*a*, *b*). As with *Carcinus*, the carpopodite extensor and flexor muscles were studied and each muscle was prepared for analysis in the form of a group of washed and separated single fibres. The analyses were thus unaffected by contamination from surrounding blood and so the results could always be directly expressed in terms of intracellular concentrations. Analytical methods were identical with those used previously.

## RESULTS

(1) *The composition of the normal muscle fibres*

The concentrations of the most important osmotically active substances in the muscle fibres of crabs taken from their normal environment is shown in Table 1. The ionic composition of the muscle shows many close resemblances with that of

Table 1. *The composition of normal muscle fibres*

Substance	Concentration (mm./kg. water)	Standard deviation	Number of measurements
Potassium	111	21	8
Sodium	44	7	9
Chloride	32	3	5
Inorganic + arginine phosphate	65	—	3
Adenosine triphosphate	13	—	3
$\alpha$ -amino-N compounds	170	45	9
$\alpha$ -amino-N compounds, excluding arginine (min. concentration)	105	—	—
Trimethylamine oxide	42	—	3
Total measured osmotic activity	412	—	—
Osmotic activity of blood (m-osm./l.)	500	—	—

the muscles of marine crustacea, like *Carcinus* (Shaw, 1955*a, b*; 1958*b*) and *Nephrops* (Robertson, 1957). It is characterized by high concentrations of potassium and organic phosphate compounds but relatively low concentrations of sodium and chloride. As in the marine forms, the balance between the measured cations and anions is satisfactory: in *Potamon* the cation charge is 155 m-equiv./kg. water compared with the anion charge of 145 m-equiv./kg. water at pH 7.0. Compared with typical muscle analyses of fresh water or terrestrial vertebrates, this fresh-water crustacean differs in that the concentrations of sodium and chloride, although low compared with those in the blood, do make a significant contribution to the osmotic activity of the muscle fibre.

The similarity in the ionic composition of the muscle in *Potamon* and *Carcinus* extends beyond a mere qualitative likeness in the relative concentrations of the constituent ions: the concentrations of ions in *Potamon* muscle show a remarkable similarity to those in the muscles of *Carcinus*, taken from 40% sea water, and this extends also to the water content of the muscle. In fact, if *Potamon* has evolved from a marine decapod, similar to *Carcinus* and with a similar muscle composition, then in the course of its history there has been little change in the muscle composition from that which existed at the time of the initial penetration of brackish water by its ancestors. There has been no return to the original potassium or phosphate concentrations nor a re-establishment of the normal water content.

The inorganic ions and the organic phosphate compounds account for not much more than 50% of the total osmotic activity of the fibre, if this is assumed to be iso-osmotic with the blood. The total contribution from muscle ions may be a little greater if all ions, such as calcium, magnesium, bicarbonate, etc., were to be included, but there is a deficit of, at least, 200 m-osm./kg. water. This is largely accounted for by the measured organic compounds, the amino acids and trimethylamine oxide, which together account for at least 167 mM./kg. water. The remaining deficit may take the form of betaine, which was found in large quantities in lobster muscle (Kermack, Lees & Wood, 1955) or it may be some as yet unidentified substance. The total concentration of the non-protein nitrogenous substances in the muscle is much smaller than in the marine forms. Thus in *Carcinus* muscle the total concentration of the amino acids with taurine and trimethylamine oxide, but excluding arginine is 524 mM./kg. water (Shaw, 1958*a*) compared with the 167 mM./kg. water in *Potamon*. This lower value is probably characteristic of the fresh-water decapod Crustacea. The total free amino-acid concentration of two other fresh-water crustaceans, *Astacus fluviatilis* and *Eriocheir sinensis*, has been estimated at 153 and 239 mM./kg. water, respectively (Camien, Sarlet, Duchâteau & Florkin, 1951) and these are of the same order as found in *Potamon*.

## (2) *The composition of the muscle fibres of animals from artificial sea-water solutions*

For the purposes of these experiments animals were maintained in a standard artificial sea water made up in the following manner. About 1 l. of the solution was made by mixing 801.9 ml. of 0.6 M-NaCl, 17.2 ml. of 0.6 M-KCl, 27.2 ml. of

0.4 M-CaCl<sub>2</sub> and 139.6 ml. of 0.4 M-MgCl<sub>2</sub>. The sodium concentration of the final solution was 490 mm./l. and the potassium concentration, 10.4 mm./l.

The experimental solutions were prepared from this standard solution in the following dilutions: 100, 75, 50 and 25 %. The animals remained in the last three solutions for at least 7 days and in the 100 % solution for 3 days. After removal the muscles were analysed for water content, acid-soluble phosphate compounds, potassium, sodium and free amino acids (actually, non-protein  $\alpha$ -amino-N compounds). Penetration of salts from the higher sea-water dilutions caused increases in the blood concentration and the effect of these changes on each of the muscle constituents has been studied.

(a) *Water content*

The water content of the muscles was calculated from the difference between the wet and dry weights and thus the weight of water in the muscle was measured. The water contents, expressed as a percentage of the wet weight of muscles of animals from the different external solutions are shown in Table 2.

Table 2. *The water content of muscles from normal animals and from animals kept in sea-water solutions*

External solution	Mean water content of muscle (% wet weight)	Range of water content (% wet weight)
Lake water	78.4	75.0-83.0
25 % sea water	78.0	76.5-79.2
50 % sea water	76.7	75.2-77.9
75 % sea water	75.1	73.2-77.2
100 % sea water	70.3	67.5-75.0

A simple hypothesis to account for the effects of increased blood concentration on the water balance of the muscle fibre is that the cell functions as a simple osmometer. In this case, the amount of water withdrawn from the cell is related to the increased blood concentration in a definable manner. Thus, if the normal blood concentration =  $C$  and if the blood concentration is increased to  $nC$  then, assuming that (a) all the muscle water is solvent water, and (b) that there is no loss of dry substance from the muscle, the new water content of the muscle as a percentage of the original water content =  $100/n$ .

Now from the measurements of the water content of normal muscles and of muscle of crabs from the sea-water solutions it is possible to see to what extent this simple postulate is valid. The relation between the water content of the experimental muscles and that of a normal muscle can be calculated in the following way. Let  $W$  be the weight of water in a normal muscle and  $w$ , the weight of dry matter. The total wet weight =  $W + w$ . The water as a percentage of the wet weight,  $P$ , =  $100W/(W + w)$ . Now suppose that the percentage of water in the experimental muscle =  $P'$  and, assuming that it differs from a normal muscle only in a change in

water content, then  $P' = 100W'/(W' + w)$ , where  $W'$  is the new water content for  $w$  weight of dry substance. Hence

$$W' = P'w/(100 - P')$$

and the proportion of the new water content to that in the normal muscle,  $W'/W = P'w/(100 - P')W$ . Now in the normal muscle,  $w/W = (100 - P)/P$  hence

$$W'/W = P'(100 - P)/P(100 - P')$$

and the water content expressed as a percentage of the water content of a normal muscle  $= 100P'(100 - P)/P(100 - P')$ .

The water content of muscles of crabs from the four experimental solutions was calculated in this way from the measured water content and from the mean value of the water content of the normal muscle, taken from Table 2. The results are shown in Fig. 1. The points joined by the continuous line are those calculated on

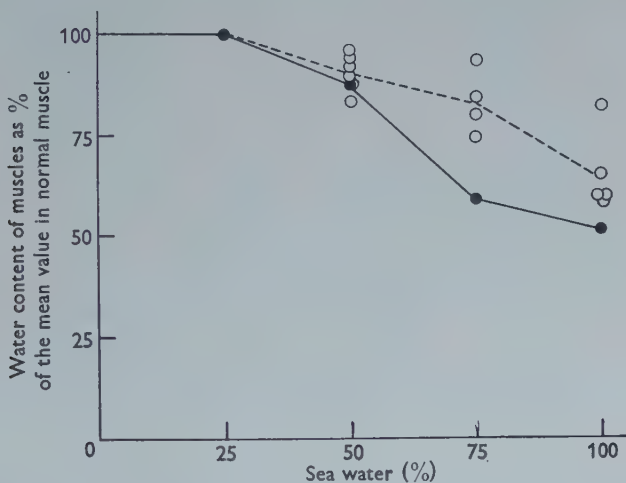


Fig. 1. The water content of muscles of animals from various dilutions of sea water. The open circles are the measured values, expressed as a percentage of the mean water content of the normal muscles. The full circles are values calculated on the assumption that the cell behaves as a simple osmometer.

the osmometer hypothesis from the mean value of the water content of the normal muscle and from the mean value of the freezing-point depression of blood of the animals from the four experimental solutions. The open circles are the actual experimental measurements of the muscle water content calculated in terms of the water content of the normal muscle (as above).

A significant divergence is apparent in sea-water concentrations greater than 50%. In both 75 and 100% solutions a considerable dehydration of the muscle has occurred, but it is not so extensive as predicted by the simple osmometer hypothesis. In 75% sea water about half the predicted water loss has occurred, but in full-strength sea water the dehydration has been more complete. The divergence

between the measured and calculated water content suggests that the osmotic activity of the muscle fibres has been increased partly by the withdrawal of water, but also partly by the addition of osmotically active solutes. In 75% sea water, the addition amounts to about 200 m-osm./kg. water, whereas in the undiluted sea water only just under half this amount has been added.

(b) *Phosphate*

The measurements of muscle phosphate were made on trichloroacetic acid extracts of dried muscle. The diluted extract was made normal with respect to hydrochloric acid and hydrolysed on a boiling water bath for 10 min. This treatment hydrolyses arginine phosphate and adenosine triphosphate so that the final solution contained the inorganic phosphate originally present together with phosphate derived from these two sources. These fractions accounted for the large majority of the acid-soluble phosphate compounds in *Carcinus* muscle (Shaw, 1958*b*). To circumvent

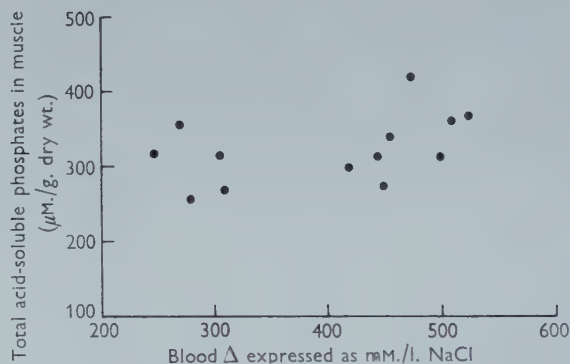


Fig. 2. The relation between the content of acid-soluble phosphate compounds in the muscle and the total concentration of the blood.

the difficulty of the change of water content of the muscles, the total phosphate concentrations were measured in terms of the dry weight and the effect of increasing blood concentrations was studied. The results are shown in Fig. 2, where the total acid-soluble phosphate content is related to the blood concentration, as measured by the freezing-point depression. It is clear that the measured blood concentrations fall into two distinct groups: the group with blood concentrations less than 350 mM./l. NaCl equiv, are from crabs in 25 and 50% sea water; the remainder are from crabs in 75 and 100% sea water. There is no indication that the muscle phosphate content has been increased following an increase in the blood osmotic pressure. A statistical test (the 't' test) for a comparison between the two groups showed no significant difference ( $t=1.7$ ;  $P$  greater than 0.05). The behaviour of the phosphate fraction appears, therefore, to be passively determined by changes in the water content of the muscle: there is no evidence of regulation of the concentration of these substances. In this respect the behaviour of the phosphate compounds is similar to that found in *Carcinus* muscle (Shaw, 1958*b*).

## (c) Potassium

Fig. 3 illustrates the relation between the potassium content of the muscles, on a dry weight basis, and the blood concentration. As with the phosphate compounds there is little evidence of a change in the potassium content of the muscles over the range of blood concentrations which have been studied. Statistically, the two groups, again, show no significant difference between their means ( $t = 2.07$ ;  $P$  greater than 0.05). As in *Carcinus* muscle, the behaviour of the potassium ions resembles that of the phosphate compounds. There is no evidence of regulation of the potassium concentration and this appears to be determined solely by water movements.

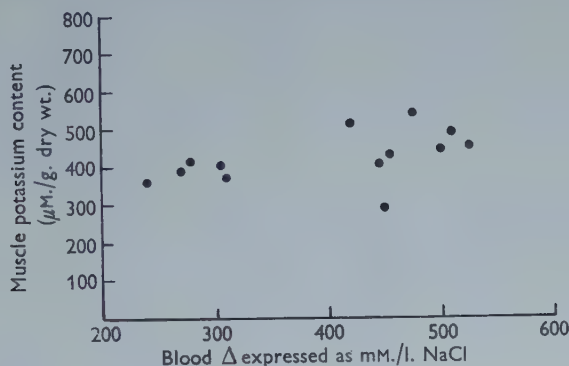


Fig. 3. The relation between the muscle potassium content and the total concentration of the blood.

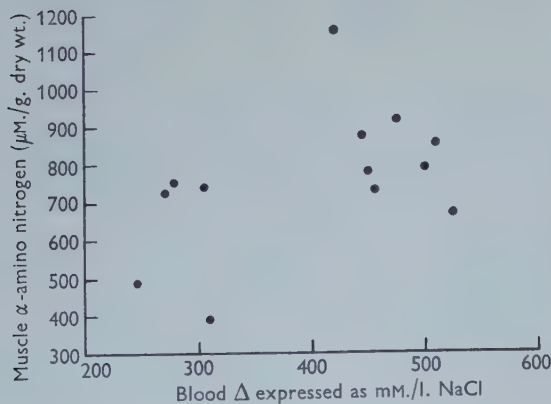


Fig. 4. The relation between the content of  $\alpha$ -amino nitrogen compounds in the muscle and the total concentration of the blood.

(d) Free amino acids and other  $\alpha$ -amino compounds

The free amino-acid content was measured on trichloroacetic-acid extracts of the dried muscle by the Folin-Danielson method for  $\alpha$ -amino nitrogen compounds. The relation between the amino-acid content and the blood concentration is shown

in Fig. 4. At the higher blood concentrations there is a small but significant increase in the amino-acid content. The difference between the means of the low and high blood-concentration groups is statistically significant, although not at a high level ( $t=2.5$ ;  $P=0.02$ ). A small quantity of amino compounds has been mobilized and added to the acid soluble fraction in response to an increased blood concentration. Table 3 shows the concentration of these compounds per kg. water content in the experimental muscles, and also the concentration which would have been expected if the change in water content had been the only operative factor.

Table 3. *The concentration of free amino acids and related compounds in the muscles of crabs from sea-water solutions*

(All concentrations are in mm./kg. water.)

External solution	Concentration	Mean concentration	Concentration calculated from change in mean water content	Amount added
25 and 50 % sea water	$\left. \begin{array}{c} 117 \\ 205 \\ 139 \\ 223 \\ 226 \end{array} \right\}$	182	180	2
75 % sea water	$\left. \begin{array}{c} 267 \\ 214 \\ 320 \\ 360 \end{array} \right\}$	290	205	85
100 % sea water	$\left. \begin{array}{c} 307 \\ 370 \\ 308 \\ 291 \end{array} \right\}$	319	261	58

The expected values have been calculated from the mean concentration in normal muscles (Table 1), the mean water content of these muscles and the mean water content of the muscles from the experimental animals (Table 2). It is noteworthy that the total concentration does not rise much above 300 mm./kg. water and that this concentration may be found in the animals from 75 % sea water. The greater part of this increase is due to the removal of water from the muscle, but a small addition to the osmotically active pool of 50–90 mm./kg. water has been made in the muscles of the animals from 75 and 100 % sea water. Since in the animals from undiluted sea water the amount added does not exceed that found in the animals from 75 % sea water where the muscle dehydration is less severe, it seems probable that this represents the maximum amount which can be brought in to boost up the normal level.

(e) *Sodium*

Fig. 5 shows the sodium content of the muscles calculated on a dry-weight basis and the relation to the total concentration of the blood. At the higher blood concentrations there is a considerable increase in the sodium content of the muscle.

The difference between the means of the high and low blood-concentrations groups is a highly significant one ( $t=5$ ;  $P$  less than 0.01). Sodium has penetrated into the muscle as a result of the increased blood sodium concentration. In *Carcinus* muscle it was found that the muscle sodium was probably present in a region of the muscle, where it was freely exchangeable with the sodium of the blood and did not penetrate into the fibre interior (Shaw, 1958*b*). The concentration of sodium in the muscle was approximately proportional to that of sodium in the blood

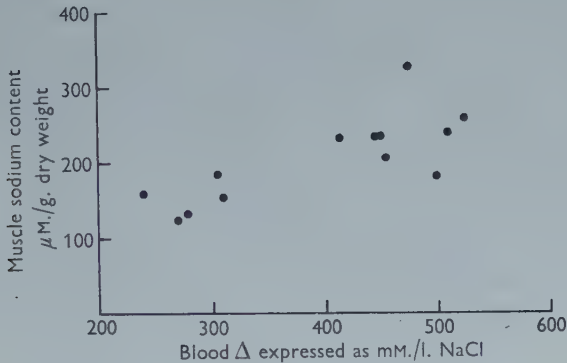


Fig. 5. The relation between the muscle sodium content and the total concentration of the blood.

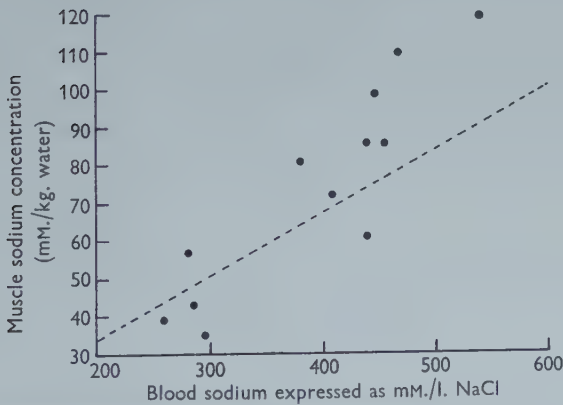


Fig. 6. The relation between the muscle sodium concentration and the blood sodium concentration. The dotted line represents a simple proportion between the two concentrations; it passes through the origin and through the mean sodium concentration of the normal muscle at the normal blood sodium concentration.

(Shaw, 1955*b*). In Fig. 6, the relation between the sodium concentrations of muscle and blood in *Potamon* is shown. The dotted line in the figure represents a linear relation between the two variables and passes through the origin and through the normal mean sodium concentration of the muscle (44  $\text{mM./kg. water}$ ). It is possible that *Potamon* muscle behaves like that of *Carcinus* at blood sodium concentrations not exceeding 450  $\text{mM./l.}$  At higher sodium concentrations the

penetration is greater than can be explained in terms of a simple proportionality with the blood sodium concentration. It is probable that at these high concentrations penetration of sodium into the fibre interior takes place.

#### DISCUSSION

Since potassium, phosphate compounds, nitrogenous substances and sodium contribute largely to the total osmotic activity of the muscle fibre, it is possible to discuss the behaviour of these substances in relation to the problem of the adaptation of the muscle cell to conditions of increased blood concentration. Potassium and phosphate behave passively with respect to water movements, make no contribution towards cell adaptation and are not themselves regulated. Unlike those of *Carcinus* (Shaw, 1958a), the muscle fibres of *Potamon niloticus* do not possess the ability to vary the content of free amino acids and related compounds over a very large range. Since the amount of these substances which can be added does not exceed 50–90 mM./kg. water, little increase in the osmotic activity of the fibres can be produced by this means. It is, however, interesting to find that some increase is possible and this may well be a legacy from the earlier marine ancestry of these animals.

Apart from increasing the amino acid content of the muscles, osmotic equilibration with the blood is achieved by the osmotic withdrawal of water and by the penetration of sodium (probably with chloride) into the fibre. In animals from 75% sea water the penetration of sodium is probably restricted to those spaces where free exchange of sodium is normally possible and the breakdown of the mechanism excluding sodium from the fibre interior does not occur. The addition to the amino-acid pool is sufficient to prevent excessive dehydration of the fibre and this probably accounts, in part, for the difference between the predicted and measured water contents of these muscles (Fig. 1). However, the added amino compounds (about 80 mM./kg.), together with the extra sodium (about 30 mM./kg.) and probably an equal amount of chloride, falls somewhat short of that required to account for the increased osmotic activity, and a small amount of some unknown osmotically active substance may also be added.

In the muscles of animals from full-strength sea water there is no further addition of free amino acids or sodium and the additional increase in blood concentration causes extensive dehydration of the muscle and the penetration of sodium into its interior. These changes may be highly significant in view of the fact that the animals are unable to survive in 100% sea water, although they can do so in the lower concentrations.

The cause of death in full-strength sea water is at present unknown. It seems unlikely that it is due to general physiological causes: for example, variations in the ionic composition of the sea water have little effect on the animals and, similarly, the termination of urine production (which has often been advanced as an explanation of the death of other fresh-water animals under similar circumstances) can hardly be important in the case of *Potamon* over a short period, since the normal rate

of excretion is extremely slow (Shaw, 1959). It is not unreasonable to suppose that the lethal effect of sea water is due to the action of the raised blood concentration on the cells, although whether all cells are affected in the same way or whether some are more adaptable than others cannot yet be surmised. If the behaviour of the muscle cell is typical of all other cell types, it may well be that the degree of cell dehydration and the maintenance of the normal sodium-excluding mechanism are factors which determine the correct functioning of the cell.

#### SUMMARY

1. An account is given of the normal composition of the muscle fibres of the fresh-water crab, *Potamon niloticus*.

2. The effect of various dilutions of sea water is considered in relation to the concentrations of a number of the muscle constituents.

3. The water content of the muscle decreases as the blood concentration is increased, but at the higher concentrations the muscle dehydration is not so extensive as expected. There is evidence of a small addition of osmotically active substances to the muscle.

4. Potassium and muscle phosphate compounds behave passively and their concentrations are determined by the muscle water content.

5. The concentration of free amino acids and related compounds may be increased beyond that due to water loss. The addition of these substances to the muscle is not greater than 50–90 mM./kg. water.

6. Sodium enters the muscle when the blood sodium concentration is increased. At blood concentrations not exceeding 450 mM./l. sodium probably only penetrates into a freely exchanging region of the fibre: at higher concentrations sodium may penetrate into the fibre interior.

7. These results are discussed in relation to the adaptability of the cell to increased blood concentrations.

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# SALT AND WATER BALANCE IN THE EAST AFRICAN FRESH-WATER CRAB, *POTAMON NILOTICUS* (M. EDW.)

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## INTRODUCTION

Among the Crustacea there are a number of fresh-water forms, of which the crayfishes are the best known examples, which are typical fresh-water animals in the generally accepted sense. The features which characterize an animal as such are: (a) the fact that it cannot survive in sea-water solutions much stronger than its normal blood concentration, (b) a body surface relatively impermeable to salts, (c) a relatively low blood concentration, and (d) the production of a dilute urine. However, there are other fresh-water crustaceans which do not possess all of these characteristics. Examples of these are the crabs, *Eriocheir sinensis* and *Telphusa fluviatile* (= *Potamon edule*) (Duval, 1925; Schlieper, 1930; Schlieper & Herrmann, 1930; Scholles, 1933) and the prawn, *Palaemonetes antennarius* (Parry, 1957). The two crabs have a high blood concentration and produce an iso-osmotic urine. *Eriocheir* is also relatively permeable to salts (Krogh, 1938) and so is the prawn, which also produces an iso-osmotic urine. Both the crabs are able to survive in full-strength sea water.

It is perhaps not surprising that the discovery of these facts concerning the fresh-water Crustacea has led to the view that whereas the crayfishes are physiologically well-adapted to life in fresh water, the other forms are not. This is supported by the fact that *Eriocheir* has to return to the sea to breed and it may well be that the other two are only relatively recent invaders of fresh water.

Beadle (Beadle & Cragg, 1940; Beadle, 1943) has suggested that the invasion of fresh water by the Crustacea has proceeded in two stages. The first stage, represented by *Eriocheir* and *Telphusa*, is characterized by the development of a mechanism for the maintenance of a high blood concentration in the dilute external medium. The second stage, represented by the crayfish, is characterized by the reduction in the blood concentration and the production of a dilute urine. As Beadle points out, our knowledge is, as yet, based on very few species and even on these, physiological studies in some cases are far from complete. It is therefore of interest to investigate other fresh-water species in the hope that this may throw further light on those factors which are important in the efficient adaptation of the animals to fresh water.

In the tropical regions of the world fresh-water crabs of the family Potamonidae are generally well established. The family is represented by a very large number of genera and species and the animals are found in a great variety of habitats. In most

parts of Africa crabs of the genus *Potamon* and of the allied genus *Potamonautes* are extremely abundant and to be found in most fresh waters. There is no evidence of their recent arrival from the sea, nor is there any reason to suppose that they are in the process of establishing themselves in the inland freshwaters. They are clearly a well-adapted and successful group by any standards. In many respects the tropical Potamonidae take the place of the crayfishes of the more temperate regions and must surely be judged as successful, if not more so, in their own geographical locations as the latter group in theirs. Therefore a comparison of the mechanisms of salt and water balance in the crayfishes and the potamonids is of considerable interest. Any differences between the two groups in this respect cannot be regarded as being due to either of them being in the process of adaptation to life in fresh water, but rather due to the adoption of different solutions to the same problem.

*Potamon niloticus* is a member of the Potamonidae which abounds in the lakes and rivers of Kenya and the eastern part of Uganda, although it is not entirely confined to these parts. There are many other species which would equally well repay study. Indeed, the variety of habitats in which other species are found are of such a kind that their inhabitants may show interesting differences in their salt and water regulating mechanisms.

#### MATERIAL AND METHODS

The animals were collected from the shores of Lake Victoria at Entebbe and then kept in an aquarium with circulating lake water. Blood samples were obtained by pipette from a puncture of the arthroal membrane at the base of the leg. Excretory fluid was collected in the following way. The animal was held by a laboratory clamp and out of water with its anterior end upwards. While viewed through a binocular microscope, the flap closing the excretory opening was lifted with a hooked needle, a pipette inserted and the excretory fluid withdrawn. Often as much as 0.2 ml. could be collected at one time. Blood and urine samples were analysed by the following methods.

*Freezing-point depression.* This was measured by the method devised by Ramsay (1949) and the apparatus was of the same general design as that described by Ramsay & Brown (1955). Owing to the unavailability of solid carbon dioxide, the apparatus was modified to include an outer cooling jacket filled with alcohol cooled by a refrigerating unit.

*Sodium, potassium and calcium.* These were measured by means of an EEL flame photometer.

*Chloride.* This was estimated by precipitation with excess silver nitrate and back titration with sodium thiocyanate.

*Conductivity.* Measurements were made on diluted samples by means of a Mullard conductivity bridge and conductivity cell.

All the methods (with the exception of those for potassium and calcium) were checked and calibrated against a standard solution of sodium chloride.

*Creatinine and inulin.* These were estimated colorimetrically by methods given by H. W. Smith (1956, Appendix V).

*Oxygen consumption.* This was measured by a flow respirometer constructed by Prof. L. C. Beadle. The apparatus had facilities for varying the oxygen tension of the inflowing water and regulating its temperature.

Table 1. *The normal composition of the blood*

Substance	Concentration (mm./l.)	Standard deviation	Number of readings
Freezing-point depression $\equiv$ NaCl solution	271	18	5
Sodium	259	28	8
Chloride	242	20	7
Potassium	8.4	0.9	8
Calcium	12.7	—	2
Conductivity $\equiv$ NaCl solution	292	—	4

Table 2. *A comparison of the blood composition of a number of freshwater Crustacea*  
(Concentrations in mm./l.)

Animal	$\Delta \equiv$ NaCl	Na	K	Ca	Mg	Cl	Author
<i>Potamon niloticus</i>	271	259	8.4	12.7	—	242	This paper Drilhon & Portier (1939)
<i>Telphusa fluviatile</i>	340	337	8.5	18.1	—	300	
<i>Eriocheir sinensis</i>	342	—	5.1	10.0	3.5	282	Scholles (1933)
<i>Astacus fluviatilis</i>	228	152	3.1	12.0	2.5	176	Bogucki (1934)
<i>A. fluviatilis</i>	—	—	5.2	10.4	2.6	194	Scholles (1933)

## RESULTS

(a) *The normal composition of the blood*

The composition of the blood of animals taken from lake water is shown in Table 1. The characteristic features are a relatively high total concentration, accounted for largely by sodium and chloride ions and a high potassium concentration. A comparison of the composition of the blood with that of some other fresh-water Crustacea is shown in Table 2. There is an obvious general similarity in the composition of the blood of the four animals, although the blood of *Potamon niloticus* shows two features of special interest. The total blood concentration occupies an intermediate position between the very high values of the other crabs and the lower concentration of the crayfish blood. This is reflected also in the concentrations of the sodium and chloride ions. The potassium concentration of the blood, like that of *Telphusa*, is relatively high as opposed to the rather low concentrations generally found in fresh-water animals.

(b) *Survival in sea-water solutions*

The ability of the animals to withstand increases in the concentration of the external medium was investigated. The animals were each placed in about 500 ml.

of the new medium and this was changed frequently. Owing to the non-availability of natural sea water an artificial sea water was used. The experimental solutions were made up from a standard artificial sea water (as detailed in Shaw, 1959*a*) and dilutions of 25, 50, 75 and 100% were employed.

The time of survival varied with the concentration of the solution. In 25 and 50% sea water the animals appeared to be able to survive for long periods (longer than 3 weeks). In 75% sea water survival was more variable: certain individuals lived in the solution for a long time whereas others were not so successful. The survival time varied from about 7 days to more than 3 weeks. In 100% sea water the animals showed a markedly different behaviour. Survival was very poor, some animals dying within a day or so and the remainder rarely surviving for longer than about 4 days.

It is interesting to compare this behaviour with that found in the other freshwater Crustacea. It is rather similar to that of *Astacus fluviatilis*. Herrmann (1931) showed that these animals could survive immersion in about 66% sea water for long periods but died in higher concentrations in a few days. The behaviour of *P. niloticus*, however, differs rather strikingly in this respect from its near relative, *Telphusa* (*P. edule*). Duval (1925) found that this species could withstand great changes in the concentration of the external medium. It could, for example, be transferred directly from a fresh water to sea water and survive in the full-strength solution for at least a month.

In *Potamon niloticus* an attempt was made to increase the survival time in full-strength sea water. In one series of experiments the transfer to the 100% solution was made gradually. The animals were adapted first to 50% sea water, then transferred to the 75% solution for some days and finally to the 100% solution. This treatment had no noticeable beneficial effect, however, for as before the animals rapidly died off within a few days. It still remains possible that a slower adaptation over the critical concentrations range from 75 to 100% might bring about a longer survival. There was another possibility that it was the ionic composition of the strong sea water rather than the total concentration which was responsible for the lethal effect on the animals. The artificial sea water differed from normal sea water in being sulphate-free, and of the ions which were present in high concentration magnesium seemed most likely to be affected. Survival was, therefore, also tested in magnesium-free sea water solutions but no improvement was found. It seems thus most likely that the death of the animal results from an increase in the total concentration of the blood above a certain critical level. The effect of an increased blood concentration on the muscle cells of these crabs has been discussed in another paper (Shaw, 1959*a*).

The relation between the concentration of the external medium and that of the blood is shown in Fig. 1. Here the freezing-point depression of the blood, expressed as a concentration of NaCl which would depress the freezing point by the same amount, is related to the concentration of the sea water in which the animal was living. The animals from 100% sea water were removed after only 3 days in the solution and therefore had probably not reached a steady state. This probably

accounts for the fact that in some cases the blood appears to be hypo-osmotic to the medium at this concentration.

The behaviour of the blood concentration is quite typical of the majority of truly fresh-water animals: it shows a slight increase as the external concentration approaches the original blood concentration and thereafter the rise is roughly proportional to the increase in the external concentration, the blood remaining slightly hyperosmotic.

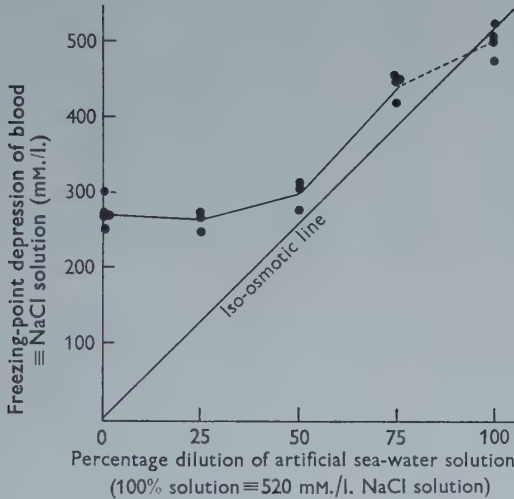


Fig. 1. The relation between the total concentration of the blood and the concentration of the external solution.

In many respects the changes in blood concentration following increases in the concentration of the external solution shown by *P. niloticus* resemble those found in *Telphusa fluviatile* (Duval, 1925). They differ in the extent to which the blood concentration can rise without causing the death of the animal.

The effect of an increase in the concentration of the external medium on some of the individual ions of the blood is shown in Table 3. There is no evidence that the animal is able to prevent the increase in concentration of any of the blood ions—in fact their concentrations are always above those of the external medium, and thus indicate a persistence of the ion uptake mechanisms which are operating to maintain the normal ionic composition of the blood when the animal is in its normal environment.

### (c) Composition of the urine

The composition of the normal excretory fluid is shown in Table 4. The fluid resembles that of *Eriocheir* and *Telphusa* in that it is practically iso-osmotic with the blood. The difference between the means for the blood and the urine freezing-point depression is barely significant ( $t=2.2$ ;  $P=0.05$ ). In three cases where blood and urine measurements were made on the same animals the ratios of the total concentration of the blood to that of the urine were 297:247, 283:260 and 265:215, respectively; the urine concentration was always slightly lower. It is possible,

Table 3. *The composition of the blood of animals from sea-water solutions*  
(Concentrations in mM./l.)

Sea-water dilution (%)	$\Delta \equiv$ NaCl solution	Na	Cl	K	Conductivity $\equiv$ NaCl solution
25	240	250	242	7.0	—
25	270	296	251	8.0	295
25	247	246	—	7.0	—
50	310	286	270	8.5	330
50	278	260	258	9.0	—
50	305	282	280	8.5	—
75	450	380	408	10.0	445
75	455	432	452	13.0	500
75	445	440	452	11.0	457
75	420	408	408	11.5	442
100	475	464	487	13.5	530
100	500	456	460	14.0	540
100	525	540	510	13.0	573
100	510	448	460	10.5	—

Table 4. *The composition of the normal excretory fluid*

Substance	Concentration (mM./l.)	Standard deviation	Number of readings
Freezing-point depression $\equiv$ NaCl solution	246	20	6
Sodium	240	33	9
Chloride	238	—	3
Potassium	3.7	1.4	9
Calcium	13.0	—	2

therefore, that the excretory fluid is very slightly hypo-osmotic to the blood. Apart from this very small difference the most obvious feature of the urine composition is the low potassium concentration compared with that of the blood. The urine potassium concentration is less than half that of the blood and this difference is statistically highly significant ( $t=7.8$ ;  $P<0.01$ ). The potassium ion may well be reabsorbed in the interests of the maintenance of a relatively high blood potassium concentration.

The composition of the urine of animals from sea-water solutions shows the same general picture. This is illustrated in Table 5 where the blood and urine concentrations are compared. As the blood concentration increases the sodium and chloride concentrations of the urine are also increased. These generally remain slightly below the corresponding concentrations in the blood. In the case of sodium there was one animal where the concentration was as low as 70% of that of the blood, but in all other cases the concentrations lay between 90 and 104% of the blood levels. The behaviour of the chloride ion was almost exactly the same as that of sodium. The concentration of potassium was still maintained at a fairly low level despite the considerable increase in the blood potassium concentration.

Table 5. *The composition of the excretory fluid of animals from sea-water solutions*  
(Concentrations in mM./l.)

Sea-water dilution (%)	Fluid	Na	Cl	K
25	Blood	296	251	8.0
	Urine	246	—	7.0
75	Blood	380	408	10.0
	Urine	395	397	2.9
75	Blood	432	452	13.0
	Urine	302	313	2.5
75	Blood	440	452	11.0
	Urine	415	380	2.2
100	Blood	464	487	13.5
	Urine	400	424	10.8
100	Blood	456	460	14.0
	Urine	415	—	3.1

(d) *The rate of urine production*

Measurements of the rate of urine production were made by the usual methods. These may be listed under three heads: (a) weight changes after blocking the excretory openings or after transferring the animals to blood iso-osmotic sea water, (b) the collection of measured amounts of excretory fluid at intervals between which the excretory openings are sealed, and (c) the injection of non-metabolized substances into the blood and the measurement of the rate of appearance of these substances in the external medium. None of these methods is ideal by itself, but when they are all used on the same animals then results obtained by their use can be interpreted with confidence. The application of these methods to a number of animals is illustrated in Table 6. The results were surprising and unexpected.

All the methods pointed to a very low rate of fluid production. The weight measurement methods were clearly not sensitive enough to allow an estimate of the urine production rate to be made. The urine collections revealed that at the most only very small amounts of fluid were being formed and the rate of production did not appear to be greater than about 0.05 % of the body weight per day. In the injection experiments, inulin and indigocarmine could not be detected in the water at all during several days, although, in the case of the dye, it could easily be demonstrated in the excretory fluid itself. The methods for the estimation of creatinine are more sensitive than those for inulin and indigocarmine and a real attempt was made by injecting creatinine solutions to obtain a quantitative measure of urine production. Unfortunately, it was found that owing to the very slow rate at which creatinine appeared in the water it was destroyed by bacterial action before a sufficiently high concentration for an accurate measurement had been built up. In certain experiments the bacterial action was prevented by keeping the animals in a diluted buffer at pH 4, although this entailed sacrificing normal physiological conditions. Under such conditions it was possible to demonstrate the appearance of

Table 6. *The rate of production of excretory fluid*

(1) Weight changes (all weights in g.)

Specimen no.	Days after blockage							
	1	2	3	4	5	6	7	8
(a) With excretory pores blocked								
1	17.8	18.1	18.3	18.3	18.3	18.3	18.1	18.4
2	33.4	33.6	33.6	33.6	—	—	—	—
3	17.7	17.7	17.6	17.8	—	—	—	—
4	14.6	14.7	14.7	14.7	—	—	—	—
(b) Bladder emptied first and then pores blocked								
5	26.9	26.9	—	—	26.9	—	—	—
6	20.3	20.0	—	—	20.0	—	—	—
(c) In 50 % sea water but pores not blocked								
7	14.1	14.0	14.0	13.8	14.1	—	—	—

(2) Urine extracted, pores blocked and returned to water.

Urine extracted again later

Specimen no.	Urine collected after 1 day (ml.)	Urine collected after 5 days (ml.)
1	0.007	—
2	0.01	0.035
3	0.02	—

(3) Injection experiments

- (a) Indigocarmine. Injected 0.1 ml. of a 1 % solution. No dye detected in the water after 3 days  
 (b) Inulin. Injected 0.1 ml. containing 5 mg. No inulin detected in the water after 3 days  
 (c) Creatinine. Injected 5 mg. Animals kept in dilute acetate buffer at pH 4

Specimen no.	Creatinine recovered in buffer solution ( $\mu$ g.)	
	1st day	2nd day
1	120	100
2	130	80

creatinine in the water after an injection into the blood. Following an injection of 5 mg. creatinine it appeared in the water at a rate of about 100  $\mu$ g./day. If it was assumed that all the creatinine came out through the excretory system, then a urine production rate of about 0.6 % body weight/day was indicated. This value is almost certainly too high, since it includes loss from all sources including leakage through the injection wound. It is possible also that the low pH of the water had an adverse effect on the permeability properties of the body surface.

It was not possible therefore to arrive at a definite measure of the rate of urine production. The creatinine experiments may be regarded as giving a maximum

value and the true rate probably lies between 0.6 and 0.05 % body weight/day. Whatever the actual value it is clear that the rate of urine production is exceptionally low and of a different order from that recorded previously for fresh-water animals.

It is possible that there is some other route for the removal of water taken up by osmosis, although such mechanisms have not been demonstrated in other Crustacea or, for that matter, in any other coelomate animals. The fact that substances injected into the blood do not readily appear in the water indicates that if such an alternative route did exist it could not involve the formation and discharge of a blood filtrate, but must presumably take the form of a mechanism for the active secretion of water. Although such a hypothetical mechanism is possible, in the absence of any evidence it seems more plausible to accept the alternative explanation that the water permeability of the body surface is very low.

(e) *The rate of loss of salts*

Although the urine is iso-osmotic with the blood, the very low rate of production insures that the salt loss through this channel is very small. Nevertheless, an appreciable loss of salts does occur and this takes place presumably through the body surface. Normally this loss is balanced by an equal uptake of salts brought about by active uptake mechanisms. The rate of loss was measured in two ways. The first method involved washing the animals in flowing distilled water at such a rate that the concentration of salts in the water itself did not build up sufficiently rapidly for an appreciable active uptake to occur. The washing water was collected and the rate of flow measured together with the sodium and potassium concentrations. From this the rate of loss of these two ions from the animal was calculated. In the second method the uptake mechanism was inhibited by bubbling through the external solution a mixture of air and carbon dioxide in the proportion of 10:1. The rate of loss of sodium was calculated by measuring the increase in sodium concentration of a known volume of the external solution. The results obtained by the use of the two methods are shown in Table 7.

The mean value for the rate of loss of sodium was  $8.0 \mu\text{M.}/10 \text{ g./hr.}$  Sealing off the excretory openings had no effect on the rate of loss over a 3 hr. period. The mean rate of loss of potassium amounted to  $0.5 \mu\text{M.}/10 \text{ g./hr.}$  Potassium was lost at about one-sixteenth of the rate of sodium loss. However, since the blood sodium concentration is about thirty times greater than that of potassium the permeability of the body surface to potassium must be nearly twice as great as to sodium.

Since the excretory organ is not involved to any appreciable extent in the loss of salts, the inorganic ions must be diffusing out through a permeable body surface. This may well be the main route for salt loss in many fresh-water Crustacea. Thus in *Eriocheir* Krogh (1938) estimated that 86 % of the salt loss occurred across the body surface and in *Astacus* Wikgren (1953) and Shaw (1959b) found that at least 90 % of the sodium and chloride loss appeared to be extrarenal. It is interesting to compare the rate of sodium loss in these three animals. In *Potamon niloticus* the sodium loss is about five times greater than in *Astacus pallipes* (Shaw, 1959b) but is rather smaller than in *Eriocheir*. No accurate measurement of salt loss has yet

Table 7. *Rate of loss of salts*

Animal	Weight (g.)	Sodium loss rate ( $\mu\text{M./hr.}$ )			Mean ( $\mu\text{M./hr.}$ )	Specific loss rate ( $\mu\text{M./10 g./hr.}$ )
		1st hr.	2nd hr.	3rd hr.		
A. Sodium loss						
Distilled water method						
1	10.2	7.8	5.8	6.8	6.8	6.7
2	19.3	15.5	12.5	—	14.0	7.3
Carbon dioxide method						
3	14.8	16.0	15.0	15.1	15.3	10.5
3*		15.6	14.5	17.0	15.7	
4	15.1	11.0	11.4	—	11.2	7.5
4*		11.8	11.7	11.0	11.5	
Mean specific loss rate						8.0
B. Potassium loss						
Distilled water method						
1	10.2	0.97	0.34	0.63	0.65	0.64
2	19.3	0.50	0.42	—	0.46	0.24
5	9.8				0.62	0.62
6	7.2	} Single reading after several hours }			0.50	0.70
7	18.9				0.92	0.49
8	13.2				0.83	0.63
9	15.1				0.61	0.40
Mean specific loss rate						0.53

\* With excretory pores blocked.

been made in the latter animal, but from figures given by Krogh (1938) it appears that the loss rate is in the order of  $30 \mu\text{M./10 g./hr.}$

#### (f) *Sodium and potassium balance*

If the animals are placed in a limited volume of distilled water salts diffuse out from them and the concentration of the external solution rises until it eventually reaches a level at which salt balance is achieved. This may be called the equilibrium concentration. The equilibrium concentration depends on the amount of salt which has been lost from the animal. After excessive salt loss the animal comes into balance at a minimum equilibrium concentration which is the lowest external concentration at which balance can be achieved. This phenomenon has been described and analysed in *Astacus pallipes* (Shaw, 1959b), where the minimum equilibrium concentration was found to be  $0.04 \text{ mM./l.}$  for sodium. It was also found that the external solution had to exceed a certain volume for balance to be maintained and this was ascribed to the effect of the accumulation of excretory products and the removal of oxygen in the smaller volumes. *Potamon niloticus* behaved in a similar manner. An animal weighing between 10 and 20 g. required about 1 l. of distilled water before balance could be achieved with certainty. In this volume the animal generally reached a steady state at its minimum equilibrium concentration. The

minimum sodium external concentration for a number of animals is shown in Table 8. The mean value is 0.05 mM./l. Na and is similar to that found for *Astacus pallipes*. In *Astacus* it was found that a loss of from 5 to 10% of the internal sodium was required in order to balance at this concentration—in *Potamon* it is probably even less. To increase the concentration of a litre of distilled water to 0.05 mM./l. requires the addition of 50  $\mu$ M. Na: in a crab of, say 15 g. this would represent a loss of only about 4% of the blood sodium.

The mean value for the potassium equilibrium concentration is 0.07 mM./l. It is interesting that this should be greater than the corresponding value for sodium. The loss of potassium necessary to produce this external concentration in a litre of distilled water (70  $\mu$ M.) is greater than the total potassium content of the blood of an average weight crab and much potassium must be released from the tissues.

Table 8. *The minimum equilibrium concentrations for sodium and potassium*

Animal	Volume of distilled water (ml.)	Sodium concentration (mM./l.)	Potassium concentration (mM./l.)
1	1000	0.080	0.055
2	1000	0.050	0.065
3	1000	0.048	0.125
4	1000	0.040	—
5	1000	0.042	0.06
6	1000	0.065	0.05
	Mean	0.054	0.071
	Lake water composition	0.50	0.10

Table 8 also shows the concentrations of potassium and sodium found in the lake water from which the crabs were collected. As in *Astacus pallipes*, the minimum equilibrium concentration for sodium is well below that found in their natural water and hence a large potential sodium uptake is normally held in reserve. This is not so for potassium: the minimum equilibrium concentration is only just below the normal lake water concentration. Apparently the rate of potassium uptake is not stimulated greatly by potassium loss and, in certain circumstances, the maintenance of the normal blood potassium concentration may become a real problem. The low concentration of potassium in the excretory fluid may well be correlated with the need for potassium economy.

#### (g) *The uptake of sodium and potassium*

Under conditions of salt balance the rate of salt uptake must just equal the rate of salt loss. Thus, the normal rate of sodium absorption must be 8  $\mu$ M./10 g./hr. Sodium uptake was demonstrated by transferring animals which were in sodium balance at their minimum equilibrium concentration, into a limited volume of a more concentrated sodium chloride solution. Under these conditions a net uptake of sodium occurred. The results of these experiments are shown in Table 9. The extent of the net uptake depended on the previous sodium loss. If the loss

Table 9

(All concentrations in mm./l. Net uptakes in micromoles.)

Animal	1	2	3	4
A. Sodium uptake				
Pretreatment	—	—	—	Washed in DW for 2 days
1st soln. { Initial concn.	0	0	0	0
{ Net uptake	-50	-50	-42	-65
{ Final concn.	0.05	0.05	0.04	0.06
Pretreatment	—	Washed in DW for 1 day	—	—
2nd soln. { Initial concn.	0.2	0.2	0.5	0.5
{ Net uptake	+116	+140	+10	+250
{ Final concn.	0.08	0.06	0.49	0.25
Pretreatment	—	—	Washed in DW for 2 days	Washed in DW for 2 days
3rd soln. { Initial concn.	0	0.2	0.5	0
{ Net uptake	-60	+86	+130	-44
{ Final concn.	0.06	0.11	0.37	0.04
Pretreatment	—	Washed in DW for 4 days	—	—
4th soln. { Initial concn.	0.50	0.50	—	0.50
{ Net uptake	+90	+440	—	+410
{ Final concn.	0.41	0.06	—	0.09
Pretreatment	—	—	—	—
5th soln. { Initial concn.	—	0	—	0
{ Net uptake	—	-55	—	-50
{ Final concn.	—	0.05	—	0.05
B. Potassium uptake				
1st soln. { Initial concn.	0.2	—	—	0.2
{ Net uptake	+47	—	—	+48
{ Final concn.	0.15	—	—	0.15

DW = Distilled water.

was great (as, for example, occurred when the animal was washed in running distilled water for several days) then sodium net uptake continued until the external concentration was reduced to near the original minimum equilibrium concentration (see animals 2 and 4 in Table 9). The original equilibrium concentration was not reduced by further sodium loss.

The similarity in the behaviour of *Potamon niloticus* and *Astacus pallipes* (Shaw, 1959b) with respect to sodium balance and sodium uptake is striking—they probably differ only in the magnitude of the fluxes. If, as it appears, the sodium uptake mechanism in *Potamon* is influenced in the same way as in *Astacus* by external and internal sodium concentrations, then their effect may be represented quantitatively as in Fig. 2. The value for the maximum rate at 0.5 mm./l. (16  $\mu$ M./10 g./hr.) was calculated from the sum of the maximum observed net uptake rate (8  $\mu$ M./10 g./hr. by animal 4, fourth solution, Table 9) and the normal loss rate (8  $\mu$ M./10 g./hr.). These relations account for the observed sodium movements. Thus sodium balance

normally occurs at an external concentration of  $0.5 \text{ mM./l.}$  (curve 1, Fig. 2). A loss of  $50 \mu\text{M. Na}$  shifts the uptake curve to its maximum position (curve 2) and the animal will be in sodium balance at an external sodium concentration of  $0.05 \text{ mM./l.}$  If the animal is now placed in  $0.5 \text{ mM./l. NaCl}$ , the sodium uptake mechanism will be working near its maximum rate and a net uptake of sodium will take place. However, as soon as about  $50 \mu\text{M. Na}$  has been absorbed the uptake rate will adapt back to its original level and balance will be restored. If the animal had lost a large amount of sodium and was then placed in  $0.5 \text{ mM./l. NaCl}$  then again a net uptake of sodium occurs, but the uptake will continue at this rate until the large sodium loss has been made good. Adaptation back to the original rate will then take place as before.

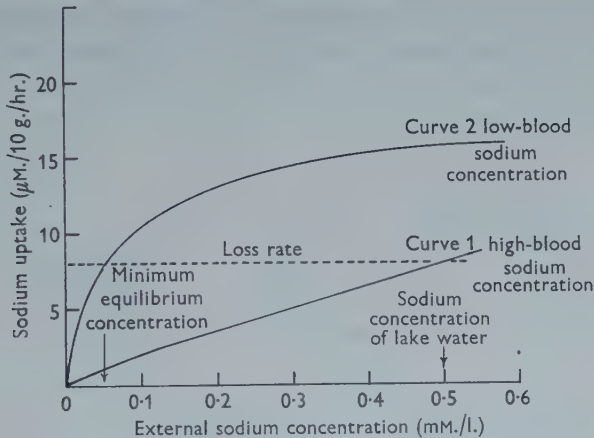


Fig. 2. A possible relation between the rate of sodium uptake and the external sodium concentration.

This type of self-regulating sodium balance system may well turn out to be of common occurrence among the fresh-water Crustacea.

Potassium uptake was not studied in detail. Two experiments, shown in the lower part of Table 9, show that a net uptake of potassium does take place when the external concentration exceeds the equilibrium concentration. It is worth noting that at low external concentrations the sodium uptake rate may be sixteen times that of potassium despite the fact that the concentration gradients very much favour potassium absorption.

#### (h) The effect of environmental factors on the rate of sodium uptake

Fresh-water animals which are dependent on salt uptake mechanisms for the maintenance of salt balance may be limited in their distribution by environmental factors which may depress the activity of these mechanisms. For example, factors such as the salt content of the water, temperature, oxygen content of the water, pH may all be of great importance. Among the fresh waters of East Africa conditions may be found where any one of these factors might be significant in determining the distribution of the crabs. Although no detailed investigation was made of the

effect of these factors on salt uptake, a number of observations indicate their probable importance and suggest some interesting lines for eco-physiological studies in the area.

The importance of the salt content of the water has already been mentioned. *Potamon niloticus* cannot maintain sodium balance in a concentration less than 0.05 mM./l. Na or potassium balance in a concentration less than 0.07 mM./l. K. Very soft waters are, however, found in the area with concentrations as low as this. For example, the River Sezibwa water (below the Sezibwa Falls, August 1957) contained only 0.11 mM./l. Na and 0.02 mM./l. K. Although the sodium concentration is just high enough to maintain sodium balance in *P. niloticus* the potassium concentration is too low. It was therefore interesting that no specimens could be collected from here although another species (probably *P. johnstoni*) was present. In a few laboratory experiments it was found that this species had a considerably lower minimum equilibrium concentration for sodium than *P. niloticus* (it was between 0.01 and 0.02 mM./l. Na).

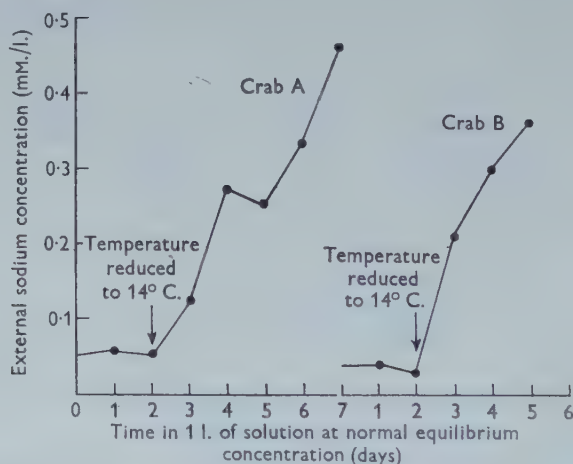


Fig. 3. The effect of a reduction in temperature on the normal equilibrium concentration at 24°C.

The technique of bringing an animal into sodium balance in a limited volume of distilled water was used to demonstrate the effect of two other factors, temperature and oxygen tension, on the sodium uptake in *P. niloticus*. The animal was first allowed to come into balance and maintain it for a few days. Then either the temperature was reduced or the water was saturated with a nitrogen:air mixture and the effect of these new conditions on the external sodium concentration was followed. These experiments are illustrated in Figs. 3 and 4. In the case of the temperature change, a reduction of 10°C. upsets the balance and the external sodium concentration increases steadily.

It was of great interest to find an example of the possible limitation by temperature of the distribution of *P. niloticus* in the field. In Eastern Uganda the River Manafwa arises from near the top of Mount Elgon and flows down into Lake Victoria. Along

the river there is a temperature gradient in the water from near  $0^{\circ}\text{C}$ . at the source to  $20\text{--}30^{\circ}\text{C}$ . at its lower reaches. *P. niloticus* was found in large numbers in the lower parts of the river. The river was followed for some distance up Mount Elgon and over this section the midday temperature of the water gradually fell from  $20$  to  $9.5^{\circ}\text{C}$ . at the highest point. The crab became progressively scarcer and where the temperature had dropped to about  $13^{\circ}\text{C}$ . no *P. niloticus* could be collected. Water analysis of samples taken at various places up the river showed that the water had not changed appreciably in composition. Again it was fascinating to find that as *P. niloticus* disappeared it was replaced by another species, *Potamonautes granviki*, which was found up to the highest point. This species has also been recorded from the top of Mount Elgon in water whose temperature was about  $2^{\circ}\text{C}$ . (Prof. L. C. Beadle, private communication).

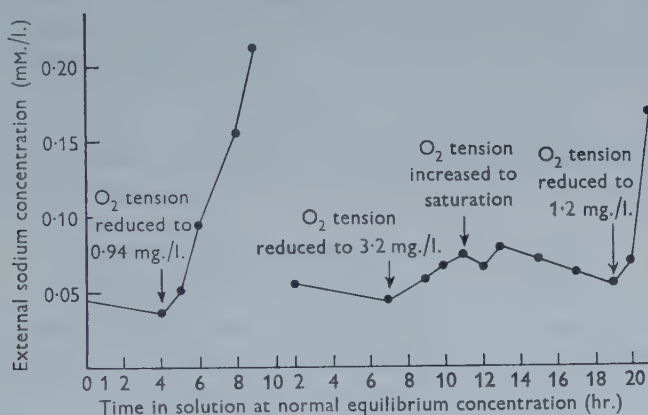


Fig. 4. The effect of a reduction in oxygen tension of the water on the normal equilibrium concentration.

The effect of oxygen tension of the water on sodium balance is shown in Fig. 4. A reduction of the normal oxygen tension by half has very little effect on the sodium balance providing the supply of oxygen is sufficiently rapid. At lower tensions the animals go out of balance and the external sodium concentration rises.

Over much of Uganda there are large areas of swamp which border the edges of the lakes or form part of the river systems. In these regions oxygen tensions are invariably low (Carter, 1955). Again, it is interesting to find that *Potamon niloticus* is apparently unable to live in these conditions. Prof. L. C. Beadle has made extensive investigations of the swamps bordering the shores of Lake Victoria but has never found crabs in these areas, despite the fact that the animals are found abundantly on the rocky shores of the lake (private communication).

These preliminary ecological studies show that although *P. niloticus* is widely distributed there are a number of ecological situations in which it is apparently unable to survive. There may, of course, be many factors which determine its distribution, but it is certainly possible that the provision of suitable conditions for the maintenance of salt balance may be one of the most important.

(i) *Oxygen consumption*

The normal oxygen consumption of animals in lake water was measured in order to assess the percentage of the total metabolism involved in the maintenance of salt balance. At the same time the effect of the two environmental factors, temperature and oxygen tension of the water, on the general metabolism of the animal was studied. The results are shown in Table 10. The mean value for the normal oxygen consumption at 24° C. is 0.72 mg./10 g. body weight/hr. Similar values were found by Schwabe (1933) for *Eriocheir* (0.67 mg./10 g./hr.) and *Carcinus* (0.90 mg./10 g./hr.).

Temperature has a marked effect on the oxygen consumption of *Potamon*: a reduction of 10° C. depresses it to about one-third of the normal value (Table 10). The effect of a reduction of the oxygen tension of the water may also be pronounced. The oxygen consumption is relatively unaffected by a fall in the oxygen tension to about half of the saturation value, but below this it falls off rapidly and is reduced to about one-third of normal at oxygen tensions around 1 mg./l. Since the effect of temperature and oxygen tension on sodium balance is paralleled by their effect on the oxygen consumption, it is probable that the depression of sodium uptake by these factors is not specific but is a result of the general fall in the metabolic level.

## DISCUSSION

When the mechanisms of salt and water balance in *Potamon niloticus* are considered as a whole it is apparent that the animal only resembles a 'typical' fresh-water animal in one respect: namely, its inability to survive in the higher concentrations of sea water. At first sight it may appear that *Potamon* is ill-adapted to life in fresh water in that: (a) it has a relatively high blood concentration, (b) it is relatively permeable to salts, and (c) it produces an iso-osmotic urine. However, if one compares it with other brackish-water and fresh-water crabs, such as *Carcinus* and *Eriocheir*, it becomes apparent that this view is not really tenable; in fact, *Potamon* is an example of another type of fresh-water adaptation which differs in certain ways from that shown, for example, by the crayfish. The most important factor in the adaptation of *Potamon* to fresh water has probably been an all-round reduction in the permeability of the body surface, both to salts and also to water. A comparison between the three crabs, shown in Table 11, brings this out. Here the rate of loss of sodium, the active uptake rate, the minimum osmotic work done in recovering the lost salts and the minimum percentage of the total metabolism involved in osmotic work are compared for these species. The figures for *Carcinus* are taken from unpublished data. Sodium loss in *Eriocheir* was calculated in two ways: the higher figure was derived from the loss through the urine (calculated from its concentration and the rate of urine production; Scholles, 1933) and from the fact that the urine loss represents only 14% of the total loss (Krogh, 1938); the lower figure was calculated from the initial rate of loss of sodium into distilled water (Krogh, 1938). The minimum thermodynamic work for the recovery of salts was calculated from the relation  $W = URT \ln Na_i/Na_o$ , where  $Na_i$  and  $Na_o$  are the

Table 10. *The rate of oxygen consumption*

Animal	Weight (g.)	Oxygen tension (mg./l.)	Temp. (° C.)	Oxygen consumption (mg./hr.)	Specific O <sub>2</sub> consumption (mg./10 g./hr.)
1	16.6	6.6	24	1.13	0.68
		4.0	24	1.12	—
		3.1	24	1.02	—
		1.9	24	0.53	—
2	19.1	6.8	24	1.46	0.76
		3.6	24	1.21	—
		2.0	24	0.52	—
		1.8	24	0.57	—
		1.0	24	0.43	—
		6.7	14	0.66	—
3	20.6	6.6	24	1.51	0.73
		4.9	24	1.66	—
		3.0	24	1.01	—
		2.9	24	1.02	—
		1.2	24	0.64	—
		1.2	24	0.43	—
		6.7	14	0.42	—
		6.7	14	0.31	—
Mean specific oxygen consumption at 24° C.					0.72

Table 11. *A comparison of the rate of loss of sodium and the minimum osmotic work in three crabs*

Species	Sodium loss rate (μM./10 g./hr.)	Blood Na concentration (mm./l.)	Na uptake rate (μM./10 g./hr.)	Minimum osmotic work (cal./10 g./hr.)	% total metabolism
<i>Carcinus maenas</i> (in 40 % sea water)	95	300	36	0.022	0.9
<i>Eriocheir sinensis</i> (in fresh water)	20-40	300	c. 30	0.23	10
<i>Potamon niloticus</i>	8	259	8	0.059	2.3

internal and external sodium concentrations, respectively,  $W$  is the work done and  $U$  is the uptake rate. It was assumed that chloride values were in all cases the same as the sodium ones and the total work was therefore taken as twice the sodium work. Some interesting points emerge from this comparison. *Eriocheir* is three or four times less permeable than *Carcinus*, but nevertheless it uses a very large amount of its metabolic energy for the maintenance of salt balance. This difference in permeability is, however, of great functional importance. Although *Carcinus*, in 40 % sea water, uses less than 1 % of the total available energy for the purposes of salt balance, if this crab were to move into fresh water without reducing its permeability then the work required to maintain its blood concentration would be over 30 % of its total metabolism—surely an intolerable burden. If a crab like *Carcinus* were to penetrate into fresh water this would have to be accompanied by a reduction in

permeability and, at the same time, a development of the uptake mechanism to operate at low external concentrations. Such a stage would then be similar to the situation found in *Eriocheir*. *Potamon niloticus* can be looked upon as illustrating a further development in this direction. With a permeability still lower than in *Eriocheir* (it is twelve times less permeable than *Carcinus*) the load on the total metabolism is further reduced to a reasonably low level. The special feature of *Potamon* is that the low permeability extends both to water and to salts, and with the result that salt loss through the urine is extremely small despite the fact that the fluid is iso-osmotic with the blood.

As a result of some theoretical calculations on hypothetical semipermeable animals, Potts (1954) reached the conclusion that the most important means by which an animal in fresh water can reduce the strain on its osmoregulatory mechanisms is by reducing the blood concentration and by producing a dilute urine. At first it may seem peculiar that *P. niloticus*, a highly successful fresh-water animal, should display neither of these features. The explanation lies in the fact that the basic assumption behind Potts's calculations (namely, that animals are semipermeable) seems to be of very doubtful validity. It is certainly not true of *P. niloticus*; nor is it true of *Eriocheir sinensis*, which Potts uses as an example to illustrate his arguments. Krogh (1938, 1939) explicitly states that in this animal only about 14% of the total salt loss occurs through the urine. This was also shown by Koch & Evans (1956) who found substantial sodium loss from animals with their excretory pores sealed.

If an animal is not in fact semipermeable then it is clear that the total osmotic work done in salt uptake will be greater than anticipated on this hypothesis; but a more important fact is that the conclusions which Potts arrived at for semipermeable animals are not valid for permeable ones. Thus, for example, if an animal like *Eriocheir*, where nearly 90% of the salt loss occurs through the body surface, were to produce a dilute urine, the saving in osmotic work could not exceed 10% of the total and, in fact, would be less. The same also applies to the importance of the blood concentration: if the animal is salt-permeable then the osmotic work will be approximately proportional to the blood concentration when the animal is in the fresh water (actually it is proportional to  $(B-M) \ln B/M$ , using Potts's symbols). In the semipermeable animal, on the other hand, the work is approximately proportional to the square of the blood concentration (see Potts's equations 7 and 9) and it is due to this that the blood concentration appeared to be of such special importance.

It is now apparent that the factors whose importance Potts stresses are only of especial advantage to a fresh-water animal which has achieved a differential reduction in the permeability of its body surface, i.e. it has decreased the permeability to salts but not to water, so that it has become essentially semipermeable. This may well have been the situation in an animal like *Astacus*. Certainly the rate of salt loss in *Astacus* is much lower than in *Potamon* and yet the water permeability (measured by the rate of urine production) is much higher. It is, of course, true that the minimum osmotic work done by *Astacus* is a good deal smaller than that

done by *Potamon*, but this is due to the difference in salt permeability; if *Potamon* had reduced its salt permeability to the same level as has *Astacus* it would, in fact, do slightly less osmotic work than the crayfish, despite the fact that its blood concentration is higher and that it produces an iso-osmotic urine.

With these general considerations in mind it would seem worth while to attempt to extend Beadle & Cragg's hypothesis on the steps involved in the invasion of fresh water by the Crustacea by emphasizing the importance of permeability changes. Beadle's first stage involved the maintenance of a high blood concentration in dilute solutions. This must be achieved by a reduction in permeability of the body surface, together with the development of the salt uptake mechanisms. The next step involves a further reduction in the permeability. If this takes the form of an all-round reduction in permeability to both water and salts, as in *Potamon niloticus*, then there is little to be gained by a decrease of either the blood or the urine concentration. If, on the other hand, the reduction in permeability is restricted largely to salts and the animal remains relatively permeable to water, as in *Astacus*, then the production of a dilute urine or the further reduction in the blood concentration would have a pronounced selective value.

#### SUMMARY

1. The mechanisms of salt and water balance in the East African fresh-water crab, *Potamon niloticus*, have been investigated.
2. The freezing-point depression of the blood is equivalent to that of a 271 mM./l. NaCl solution.
3. The animals cannot survive in solutions more concentrated than 75% sea water. Above the normal blood concentration, the blood osmotic pressure follows that of the medium.
4. The urine is iso-osmotic with the blood and is produced at a very slow rate. The potassium content is only half that of the blood.
5. The animal loses sodium at a rate of 8  $\mu$ M./10 g./hr. mainly through the body surface. Potassium loss occurs at one-sixteenth of this rate.
6. Sodium balance can be maintained at a minimum external concentration of 0.05 mM./l. Potassium requires a concentration of 0.07 mM./l.
7. Active absorption of both sodium and potassium occurs. The rate of uptake of sodium depends on the extent of previous sodium loss. The rate of sodium uptake may be affected by such environmental factors as the salt content of the water, temperature and oxygen tension.
8. The normal oxygen consumption rate is 0.72 mg./10 g./hr. A minimum of 2.3% is used in doing osmotic work to maintain salt balance.
9. The salt and water balance in *Potamon* is discussed in relation to the adaptation of the Crustacea to fresh water. The importance of permeability changes is stressed.

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# A NEW TONOMETRIC METHOD FOR THE DETERMINATION OF DISSOLVED OXYGEN AND CARBON DIOXIDE IN SMALL SAMPLES

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## INTRODUCTION

Four types of method are available for the determination of dissolved oxygen in biological studies, viz. chemical, polarographic, gasometric and tonometric. The last two types are also suitable for carbon dioxide determinations.

Micro-Winkler methods are satisfactory for samples of 1–2 ml. or larger, but accuracy is limited by the sensitivity of the titration end-point if smaller samples are used. Furthermore, errors arise if the samples contain certain dissolved substances such as nitrites or ferrous iron, which may occur as impurities in water samples. The latter limitation of Winkler's method has been discussed by Alsterberg (1926) and by Allee & Oesting (1934), who have proposed rather elaborate modifications to deal with specific impurities. These modifications, if found to be necessary in particular cases, considerably detract from the utility of the original method.

Polarographic methods involving carefully controlled electrolysis with the reduction of oxygen have proved useful in recent years. However, constancy of pH and of the electrolyte composition are essential and even then frequent calibration against an independent standard is necessary. The paper of Brown & Comroe (1950) and the monograph of Koltoff & Lingane (1952) should be consulted for details and references.

A number of gasometric methods are available, in which a large and definable fraction of the dissolved gases is extracted from the sample and determined volumetrically or manometrically. In the van Slyke method (van Slyke & Neill, 1924, and Oesting, 1934) the dissolved gases are extracted under vacuum and samples of about 5 ml. are required to give a reasonable standard of accuracy. Both oxygen and carbon dioxide can be determined with one sample. An alternative method (Scholander, van Dam, Claff & Kanwisher, 1955) involves extraction of the gases from the sample by mixing acid and carbonate in the sample syringe in order to generate a relatively large gas phase. This precludes the determination of carbon dioxide in the sample, but the oxygen determination can be carried out with a sample of only 1 ml. volume.

No chemical or gasometric method is applicable to the determination of the tensions\* of dissolved oxygen or carbon dioxide when respiratory pigments or buffer

\* Following Krogh the word 'tension' is used in reference to dissolved gas, the word 'pressure' in reference to the gas phase.

systems are present, since these methods will yield values for the total quantity of the respective gases.

Tonometric methods offer the most satisfactory approach to the determination of both oxygen and carbon dioxide in small volumes of biological fluids. Here the principle is simply one of equilibrating a relatively small gas bubble with the sample and subsequently analysing the bubble. Particular efforts have been made by a number of workers to evolve a satisfactory technique based on this principle, for the determination of the oxygen tension of arterial blood in man (see Riley, Campbell, & Shephard (1957) for a recent method and reference to other work). These methods commonly employ a gas bubble of about 0.03 ml. with a 1 ml. sample. Both equilibrium and absorption of the gases during the analysis are carried out in the barrel of a Roughton-Scholander syringe (1943). This syringe has a fine-bore calibrated capillary (fused on to the barrel in place of the original nozzle) in which the bubble length can be measured. The bubble remains at atmospheric pressure during the equilibration process and in consequence these methods should not be applied, for reasons which are discussed in the next section, to samples in which the total tension of dissolved gases differs from atmospheric pressure. It would appear that this limitation has been overlooked in recent years except by Roos & Black (1950). Furthermore, no attempt has been made to allow for the influence of the partial pressures in the original bubble on the final state of equilibrium. Rather has the range of application of the methods been restricted to samples whose gas tension would not be significantly altered by equilibration with a bubble of alveolar air or artificial gas mixture of fixed composition. This restriction in the case of bloods with high oxygen capacities only rules out samples in which the pigment is practically fully saturated; then the oxygen buffering power of the pigment is nil. In the case of samples lacking an oxygen buffering system such restriction of the range of application would be a severe disadvantage. Given a large enough sample/bubble ratio the buffering of carbon dioxide tension changes is never a problem because of the high solubility of this gas.

The present paper describes a tonometric method for samples of 0.3 ml. or less, giving a degree of accuracy comparable to that of micro-Winkler methods for dissolved oxygen and requiring 20–25 min. for each determination. A readily available gas mixture of constant composition (atmospheric air) is used for the bubble and the influence of the latter on the equilibrium tensions can be precisely calculated and allowed for. It is applicable to both water and blood samples in which the total tension of dissolved gases is equal to or less than atmospheric pressure, and since oxygen, carbon dioxide and nitrogen can be determined on a single sample an estimate of total tension can readily be made.

This method has the wide utility of Krogh's method (1908*a*) but uses much smaller samples. Krogh allowed a stream of the sample to pass over the bubble (volume 0.004 ml.), whose pressure was adjusted until constant volume was obtained. This required at least 15 ml. of sample, but eliminated any influence of the initial bubble partial pressures on the final equilibrium. The present method more closely resembles that of Roos & Black (1950) in its manner of equalizing total

pressure and total tension, but their method uses a sample of 17 ml. and by not allowing for the influence of the bubble its usefulness is restricted for the reasons discussed above.

#### PRINCIPLE OF THE METHOD

A small bubble of atmospheric air is enclosed with the sample in a syringe and allowed to come into diffusion equilibrium with the dissolved gases. The bubble is then transferred to the analyser where its length is measured in a uniform fine-bore capillary before and after absorption of the carbon dioxide and the oxygen by the appropriate reagents. The equilibrium partial pressures and tensions differ to a greater or lesser degree from the original tensions in the sample according to the extent of the influence of the bubble, but the initial partial tensions can be calculated from the final partial pressures in the bubble.

The micro-analysis of the equilibrated bubble, using the method of Krogh (1908*b*), presents no difficulties once the simple technique has been mastered. On the other hand, the establishment of a true equilibrium between sample and bubble calls for special care if the total tension of dissolved gases is less than atmospheric pressure. This is frequently the case in biological fluids and the extent of the 'total tension deficiency' may itself be of some interest.

Krogh (1908*a*, 1913) has called attention to the necessity for adjusting the pressure of the gas phase to equal the total tension of the dissolved gases if a true diffusion equilibrium is to be reached. If the total tension of dissolved gases is lower than the total pressure of the gas phase, gas will be continuously given up to the liquid and the volume of the gas will steadily diminish until such time as the total tension becomes equal to the total pressure. If the system is open to the atmosphere the equilibrium total tension will be equal to atmospheric pressure (i.e. liquid saturated with gas) and a relatively small bubble may well disappear altogether before equilibrium is reached. At any intermediate stage before equilibrium is reached there will be a discrepancy between the partial tension and the partial pressure of each gas. This discrepancy will be greatest for the gas with the lowest diffusion velocity.

On the other hand, if a relatively small bubble is introduced into the liquid and the system then sealed the total pressure will be quickly adjusted to parity with the total tension because the bubble-volume must remain constant. A true diffusion equilibrium is attained very rapidly with small bubbles (see discussion on 'specific surfaces'—Krogh, 1908*a*). On breaking the seal the total pressure of the bubble will return to atmospheric pressure and an instantaneous shrinkage will occur. This shrinkage will be proportional to the difference between the total tension and atmospheric pressure. Thereafter the total tension deficiency will, of course, cause an absorption of gas by the liquid as described above.

In the present method an air bubble of standard volume at atmospheric pressure is introduced into a syringe with the sample. The syringe is immediately sealed so that a true diffusion equilibrium can be reached, with the gas phase remaining at the standard volume. Afterwards the seal is broken, and before any appreciable further gas absorption can take place, the shrunken bubble is quickly transferred

to the capillary of Krogh's micro-gas-analyser (1908*b*) and its reduced volume determined. The analysis then proceeds in the normal way by successive absorption of carbon dioxide and oxygen so that, the relative proportions of these gases having been determined, the individual partial pressures can be calculated.

As Krogh pointed out (1908*a*), if the partial pressure of carbon dioxide only is required it is sufficient to equilibrate the bubble with the sample without adjusting the total pressure (or in the present method without sealing the syringe).

From a practical point of view there are considerable advantages in transferring the equilibrium bubble to a separate analyser as in the present method and in that of Roos & Black (1950). The danger of blood clots forming in the narrow analyser capillary (diameter 0.2 mm. in the present case) and the consequent necessity for the use of anti-coagulents are eliminated. Uniform drainage of the walls of the capillary, a factor which is vital to the accurate determination of the bubble volumes, is much more assured if the calibrated part is always wetted by the same solution. The latter circumstance also reduces the possibility of gas being given up to the bubble as a result of mixing air-saturated solutions of different concentrations (Krogh, 1908*b*). The amount of gas exchange during the transfer has been found to be negligible.

#### APPARATUS AND REAGENTS

Equilibration of the air bubble with the sample is carried out in an 'Agla' micro-meter syringe which has been modified as follows. The rather wide-bore glass nozzle has been replaced by one with a bore of 0.25 mm. At the inner end of this capillary nozzle a slight hollow is ground to assist in positioning the bubble below the capillary for speedy ejection from the syringe. With this capillary the dead space of the syringe is reduced to less than 0.002 ml. A 45° shoulder is ground on the tip of the nozzle, while behind this the nozzle is ground to fit a Record hypodermic needle mount. A flat brass disk carrying a pair of small hooks is cemented to the head of the syringe plunger, so that a rubber band fastened to the micrometer barrel can hold the head of the plunger firmly against the end of the micrometer screw at all times. The barrel of the syringe is clamped to the micrometer in such a position that the scale reading with the plunger right home is exactly 5 mm. This gives adequate room for applying the 'Apiezon' seal as described below. As the 'Agla' syringe is normally supplied\* with a plunger just slightly too short to reach the bottom of the barrel, it is necessary to grind a little off the rim of the open end of the barrel.

After the sample and the bubble have been introduced the syringe nozzle is sealed by applying a drop of hot paraffin wax by means of a wire loop just large enough to fit on to the shoulder of the nozzle tip. The space between the plunger and the end of the syringe barrel is sealed by a liberal application of hot 'Apiezon' grease. This is applied, by means of a fine glass jet hot enough to run immediately round the groove, as shown in Fig. 1*a*, without further heating. Wax seals are unsatisfactory in this position because they have been found to contract on setting, causing a reduction of pressure within the syringe. Furthermore, bubbles of gas tend to appear

\* By Messrs Burroughs Wellcome and Co., London.

at the interface between the wax and the water film between plunger and barrel; these must, by their expansion, bias the equilibrium between the sample and the definitive bubble. The micrometer-syringe is mounted so that it can be rotated end-over-end by a small electric motor (see Fig. 1*b*).

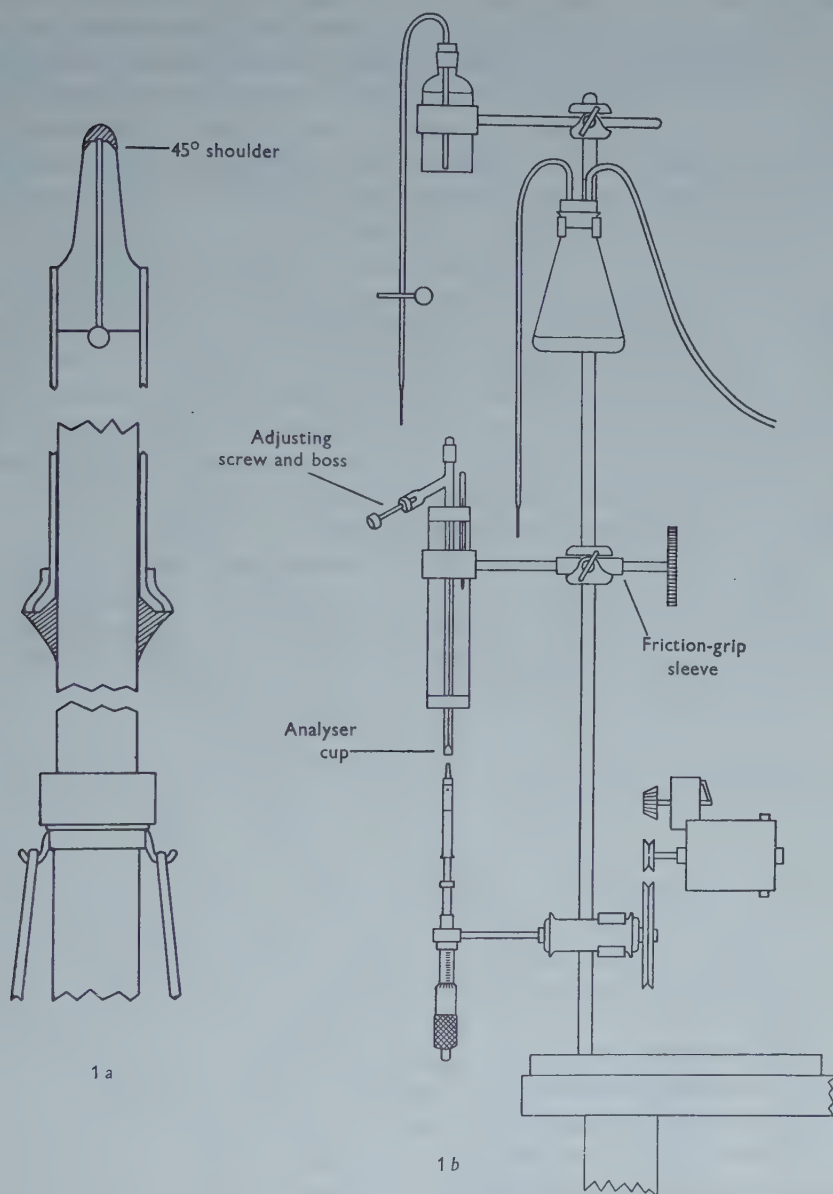


Fig. 1. Details of the tonometric apparatus. 1*a*. Parts of the 'Agla' micrometer syringe to illustrate the modifications and seals described in the text. 1*b*. Diagram of the complete apparatus (for description see text). The frame connecting the syringe barrel rigidly to the micrometer is omitted in each case.

The analysis of the equilibrated bubble in the micro-gas-analyser follows Krogh's procedure (1908*b*) fairly closely, though several points of detail have been changed. The apparatus is filled with 1% hydrochloric acid (instead of water) in order to ensure that there are no traces of alkali which could cause premature absorption of carbon dioxide. The adjusting screw and the boss in which it works are made of 'Perspex' instead of iron, to prevent any reaction with the hydrochloric acid. Finally, the alkaline pyrogallol solution for oxygen absorption has been replaced by the more satisfactory alkaline hydrosulphite solution (100 g. sodium hydrosulphite and 10 g. sodium anthraquinone- $\beta$ -sulphonate are kept as a dry stock mixture; 5 g. portions of this mixture are made up to 25 ml. in normal sodium hydroxide quickly filtered through cotton wool and stored under a layer of liquid paraffin).

The complete apparatus and the reagent bottles (1% hydrochloric acid, 10% sodium hydroxide and oxygen absorbent) are most conveniently mounted on a single 3 ft. retort stand as shown in Fig. 1*b*. At the lower end is mounted horizontally a bicycle hub whose spindle carries a pulley at one end and a clamp at the other. The complete micrometer syringe is held in the clamp so that it is roughly balanced on the spindle and can rotate end-over-end in a vertical plane at right angles to the operator's line of vision. The pulley is driven by a small rheostat-controlled motor, the speed being adjusted so that the bubble oscillates fairly rapidly from end to end of the sample in the syringe.

The analyser is mounted above at such a level that the bottom of the cup just clears the tip of the nozzle when the syringe is rotated to the vertical position. A retort clamp which grips the water-jacket of the analyser is conveniently held by a friction-grip sleeve in a retort-stand boss so that the rotation of the analyser in the vertical plane can be quickly and precisely adjusted with one hand. At the top of the stand the various reagent bottles with siphon outlets are clamped. A fine glass jet connected to a suction line is used to empty the cup of the analyser and this with its collection trap is mounted just below the reagent bottles.

#### PROCEDURE

Since the success of the method depends largely on consistent attention to a number of points of detail in the manipulation, the detailed procedure of a typical determination is given below. The apparatus and reagents having been set up as described in the previous section and the analyser having been filled with hydrochloric acid to the exclusion of all air bubbles, proceed as follows:

(1) Fill the dead space of the syringe with distilled water and screw down the plunger to the bottom of the barrel (micrometer reading = 5 mm.). Since the dead space volume is only 0.5% of the sample volume, the inclusion of the dissolved gases in this small amount of water may be disregarded. With the syringe in the horizontal position touch off the last drop of expelled water with filter-paper.

(2) Couple the syringe to the sample container without intervening air bubbles and draw back the plunger until the micrometer reads 20.1 mm.

(3) Disconnect the sample container, rotate the syringe to the vertical position (nozzle uppermost), adjust the micrometer to 20.0 mm. and touch off the drop with filter-paper.

(4) Adjust the micrometer to 20.25 mm. and then to 20.15 mm., thus drawing in *exactly* 0.003 ml. of air.

(5) On the flat end of the nozzle place a small drop of water to seal the bubble in the capillary and adjust the micrometer until the bubble is just drawn into the barrel of the syringe. Displace the bubble to one side of the nozzle and readjust the micrometer to 20.15 mm.

(6) Dry the nozzle tip and then adjust the micrometer until the water forms a tiny convex meniscus at the end of the nozzle capillary.

(7) Immediately apply a drop of molten paraffin wax to the end of the nozzle by means of the wire loop.

(8) Heat a glass dropper containing 'Apiezon' grease so that on rotating the syringe through 180° and applying the grease, the latter runs smoothly round the groove between the syringe barrel and the plunger. Enough grease should be applied to form a seal of the shape shown in Fig. 1*a*.

(9) Allow time for the grease to harden and if necessary dry outside of the syringe with filter-paper.

(10) Switch on the motor and allow the syringe to rotate end-over-end for 10 min.

(11) Switch off the motor, bring the nozzle uppermost and register the bubble in the hollow at the bottom of the nozzle capillary.

(12) Fill the cup of the analyser with a portion of the original sample (about 0.1 ml. is required). This should be omitted if the sample is a blood which clots readily and may be omitted otherwise unless the maximum accuracy is required with samples containing high partial tensions of carbon dioxide. In such cases the cup is filled with 1% hydrochloric acid.

(13) Without jolting the bubble out of the hollow, strike off the seal at the tip of the nozzle with the point of a needle and carefully expel the air drawn into the capillary by the contraction of the equilibrated bubble.

(14) Immerse the tip of the nozzle in the cup of the analyser and as quickly as possible expel the bubble. Draw the bubble into the capillary immediately. (Avoid getting fragments of wax into the cup.)

(15) Carry out the analysis of the bubble in the normal way.

(16) Before commencing the next determination clean the grease off the plunger and the barrel with filter-paper and xylol.

During the gas absorption stages of the analysis the bubble should remain in the cup only so long as is necessary for complete absorption, otherwise an appreciable amount of gas exchange may occur between the bubble and the reagents. If the amount of carbon dioxide is known to be negligible the bubble can be exposed to the oxygen absorbent directly after measuring its initial length; but if carbon dioxide is not negligible it must first be absorbed separately by exposure to 10% sodium hydroxide in the cup. Each determination then yields three measurements of the length of the bubble: before and after absorption of carbon dioxide and after

absorption of oxygen. The temperature of the sample when the bubble is introduced, the temperature of the analyser water-jacket at the time of each measurement of bubble length and the barometric pressure should all be noted. The treatment of these data is discussed in the next section.

#### CALCULATION OF RESULTS

When the total tension of dissolved gases and the individual partial tensions are close to the values for atmospheric air, it may be sufficient to deduce the total tension directly from the degree of shrinkage of the standard bubble due to equilibration, and the partial tensions from the further shrinkages due to absorption of carbon dioxide and oxygen, respectively. In most cases, however, where a reasonable degree of accuracy is required, it is necessary to take account of the influence of the bubble itself on the final state of equilibrium. By a simple algebraic treatment of the system it is possible to derive a formula (which contains the necessary correction factor) for the calculation of the partial tension of the dissolved gas.

By means of a calibration table or conversion factor, bubble lengths measured in the analyser capillary are converted to volumes. The following symbols are used:

- $V$  volume of equilibrated bubble (ml.);
- $V_1$  volume of bubble after  $\text{CO}_2$  absorption;
- $V_2$  volume of bubble after  $\text{O}_2$  absorption;
- $B$  barometric pressure at time of observation (assumed to be constant during a single determination);
- $T$  temperature of sample ( $^{\circ}\text{A.}$ ).

The above factors are measured with each determination.

- $\alpha_1$  absorption coefficient of  $\text{CO}_2$  at temperature  $T$ ;
- $\alpha_2$  absorption coefficient \* of  $\text{O}_2$ ;
- $x$  partial tension of the gas in question, in the sample *before* equilibration (mm. Hg);
- $a$  partial tension of the gas in question, in the sample *after* equilibration;
- $p$  vapour pressure of water at temperature  $T$  (mm. Hg);
- 0.3 volume of the sample (ml.);
- 0.003 volume of the standard air bubble (ml.).

The following treatment illustrates the manner in which the partial tension ( $t_{\text{O}_2}$ ) of oxygen in the original sample should be calculated. In the closed system the amount of oxygen is constant though the distribution of the gas between the gas and liquid phases alters during the equilibration. The states of partition before and after equilibration may be equated, the volumes of oxygen being measured or calculated at  $B$  mm. and  $T^{\circ}\text{A.}$

\* The absorption coefficient of a gas in a liquid is the volume of that gas (reduced to N.T.P.) absorbed by one volume of the liquid when the pressure of the gas itself amounts to 760 mm.

Before equilibration:

Air bubble of 0.003 ml.  $\equiv$  0.00062 ml.  $O_2$ .

$$\begin{aligned}\text{Sample of 0.3 ml.} & \equiv \frac{0.3\alpha_2 x}{760} \frac{T}{273} \frac{760}{B} \text{ ml. } O_2 \\ & = k_2 x,\end{aligned}$$

if  $0.3\alpha_2 T/273 B$  be represented by  $k_2$  (see below).

After equilibration:

Air bubble  $\equiv V_1 - V_2$  ml.  $O_2$ .

Sample  $\equiv k_2 a$  ml.  $O_2$

Equating conditions before and after equilibration

$$k_2 x + 0.00062 = k_2 a + (V_1 - V_2),$$

$$\text{therefore } x = \frac{k_2 a + (V_1 - V_2) - 0.00062}{k_2},$$

but  $a = t_{O_2}$  in sample after equilibration

$= p_{O_2}$  in equilibrated bubble *sealed*

$= p_{O_2}$  in bubble *after breaking seal*  $V/0.003$ ,

$$\text{therefore } a = \frac{(V_1 - V_2)(B - p)}{V} \frac{V}{0.003},$$

substituting for  $a$

$$x = \frac{(V_1 - V_2)(B - p)}{0.003} + \frac{(V_1 - V_2) - 0.00062}{k_2}.$$

The first term in this equation represents the simple proportionality between total and partial pressures and volumes in the equilibrated bubble, while the second term is the correction factor for the influence of the bubble on the final equilibrium. For the calculation of the constant  $k_2$ , the values  $\alpha_2$ ,  $B$  and  $T$  need to be known approximately. The value of the absorption coefficient  $\alpha_2$ , which is constant for one particular kind of sample at a given working temperature, may be taken as the same as for water in the case of dilute aqueous solutions. For the body fluids of most animals the value of equimolar sea water will suffice, while for other kinds of samples the solubility can, if necessary, be determined with the van Slyke apparatus.

If the sample is a blood containing a respiratory pigment the amount of contained gas will not be directly pressure-dependent. However, if the dissociation data and the oxygen capacity are known it is possible to draw the dissociation curve in the form relating volumes per cent. of combined oxygen to oxygen partial tension. If the approximate oxygen partial tension is calculated a straight line can be drawn tangential to the dissociation curve at the relevant partial pressure. By extrapolating this line to 0 and to 760 mm. the appropriate value of  $\alpha_2$  can be determined graphically. In many cases the value of  $\alpha_2$  thus deduced will give a value of  $k_2$  so large as to make the correction factor insignificant, as it is in the case of carbon dioxide (see below). The principal value of this procedure will be in dealing with blood samples which are almost fully saturated with oxygen or have very low oxygen

capacities. When the pigment is fully saturated combined oxygen is constant and only  $\alpha_2$  for dissolved gas need be considered. For pigments which are nearly saturated or have very low oxygen capacities the plasma oxygen may be a significant part of the pressure-dependent total, in which case the value of the absorption coefficient for dissolved oxygen should be added to the value deduced for the pigment by the above procedure.

Without sensible error  $T$  and  $B$  may be given fixed values in calculating  $k_2$  for a day's working, the error due to any normal variation in room temperature or barometric pressure being of no practical significance in this context.

The above formula serves equally well for the calculation of the partial pressure of carbon dioxide if the appropriate substitutions are made. Thus  $V$  is substituted for  $V_1$ ,  $V_1$  for  $V_2$ ,  $k_1$  for  $k_2$  and 0 for 0.00062 (carbon dioxide in the initial bubble can be neglected). However, because of the high solubility of carbon dioxide, the influence of the bubble on the equilibrium partial tension of the gas is very small. The correction factor has a constant value (if the carbon dioxide in the initial bubble of atmospheric air is neglected) of +1.1% of the value obtained from the expression  $(V - V_1)(B - p)/0.003$ . If the sample contains a carbon dioxide buffering system the correction factor will be quite insignificant.

The shrinkage of the equilibrated bubble after breaking the seal is proportional to the ratio of atmospheric pressure to the equilibrium total pressure (= total tension); but the latter may differ significantly from the original total tension of gases in the sample because of the influence of the gases in the initial bubble on the final equilibrium. In such cases the degree of shrinkage does not give an accurate estimate of the total tension of gases in the original sample. However, if a value for the total tension is required, it is a simple matter to repeat the above calculation for the nitrogen in the sample. The value of  $k_3$  should be calculated and the appropriate values substituted for the volume of nitrogen in the initial bubble (0.00238 ml.) and in the equilibrated bubble ( $V_2$ ). The total tension of dissolved gases is, of course, the sum of the calculated partial tensions of carbon dioxide, oxygen and nitrogen.

Before carrying out the calculations described above, the influence of any temperature variation on the measured volumes of the bubble must be allowed for. The analyser capillary should be calibrated with reference to a standard bubble measured by the micrometer syringe at the same temperature as the water-jacket of the analyser. Subsequently, for each determination four temperatures should be recorded: the temperature of the syringe and sample when the bubble is introduced (= temperature of the room air if this is not fluctuating too rapidly) and the temperature of the water-jacket at the three times of measuring the bubble in the capillary. If any of the last three differ from the first, appropriate corrections should be applied to  $V$ ,  $V_1$  and  $V_2$ . This is conveniently done by adding (subtracting) to the logarithm of the observed volume 0.00015 for every 0.1° C. fall (rise) in temperature.

In theory, account should be taken of any variation in the temperature of the sample during the equilibration period. This will alter the absorption coefficients and so cause a change in the partial tensions of the dissolved gases. At normal room

temperatures a  $1^{\circ}\text{C}$ . change will cause an approximate change of 2.8, 1.9 and 1.5% in the partial tensions of dissolved carbon dioxide, oxygen and nitrogen, respectively. It has been found that with reasonable precautions the temperature of the sample during the equilibration period can be kept constant within  $\pm 0.2^{\circ}\text{C}$ . without recourse to a water-bath. If the method is to be used with samples containing a respiratory pigment whose oxygen capacity has a high temperature coefficient more stringent temperature control may be necessary.

#### SOURCES OF ERROR AND ACCURACY

The procedure set out above has been devised to minimize a number of sources of error. Foremost among these is the excessive shrinkage of the bubble in the unsealed syringe if the total tension of dissolved gases is less than atmospheric pressure. The time during which the air bubble lies in contact with sample in the unsealed syringe or in the analyser cup must therefore be kept as short as possible and agitation kept to a minimum. The accuracy with which the standard bubble is measured and drawn into the syringe by the micrometer and the efficacy of the seals are also important factors. The standard bubble can be reproduced with an accuracy of  $\pm 1\%$  volume if the micrometer is carefully used and the capillary of the syringe nozzle is kept clean. It has been found that the sealing technique as described is entirely adequate, apart from an occasional failure of the nozzle seal. This fault reveals itself by a leakage of air into the capillary of the nozzle *before* the seal is struck off at the end of the equilibration period. It should be a matter of routine to check this point after every equilibration.

For a discussion of factors affecting the accuracy of the analysis of the bubble reference should be made to Krogh's original paper (1908*b*). It may be remarked that in the present method the pre-analysis stages in the manipulations are likely to contain the more important sources of error (excluding errors of meniscus readings). Adherence to the details of the procedure set out above and attention to the cleanliness of the syringe (especially of the capillary nozzle) will ensure a standard of accuracy consistent with the examples set out in Table 1.

Distilled water samples were equilibrated with a variety of mixtures of carbon dioxide, oxygen and nitrogen at total pressures ranging from 750 down to 510 mm. Three portions of each were subjected to complete analysis by the tonometric method, and duplicate analyses, for dissolved oxygen only, were also made on additional portions by the micro-Winkler method of Fox & Wingfield (1938). The results of these determinations are set out in Table 1. The tonometric determinations show a high degree of consistency as indicated by the coefficients of variability (column headed  $V$ ). On the basis of these values of  $V$ , replicate determinations of carbon dioxide, oxygen and total gas tension are seen to agree within 3.0, 2.2 and 1.2%, respectively. The tendency for  $V$  to increase as the partial tensions ( $\equiv$  volume differences) decrease is due, in part at least, to the fact that the possible error in reading the meniscus at each end of the bubble is absolute and assumes a greater importance as the volumes ( $V - V_1$ ) and ( $V_1 - V_2$ ) decrease. Each calculated partial

tension is based on two volume measurements, i.e. four meniscus readings. In addition, the values of  $V$  will be influenced by variations in the volume of the standard bubble, and by the extent of unwanted gas exchange between bubble and sample in the unsealed syringe. The latter factor will be of greatest importance when the oxygen tension is low and the carbon dioxide tension high. Because of differences in diffusion velocities these unwanted gas exchanges are more serious in the case of carbon dioxide than of oxygen or nitrogen. This is reflected in the higher values of  $V$  obtained with carbon dioxide determinations. Values of  $V$  for total gas tensions

Table 1. *Comparisons of determinations of dissolved gases in distilled water samples*

(Gas tensions in mm. Hg. 'Total' figures are the sum of determined tensions of carbon dioxide, oxygen and nitrogen. ' $V$ ' is the coefficient of variability—standard deviation as a percentage of the mean.)

Gas	Sample no.	Tonometric			Mean	$V$	Winkler		Mean
CO <sub>2</sub>	1	1.0	1.5	—	—	—	—	—	—
	9	9.7	9.7	9.5	9.6	1.25	—	—	—
	7	20.4	19.9	19.9	20.1	1.44	—	—	—
	6	19.5	20.2	20.7	20.1	2.98	—	—	—
	2	26.5	25.9	26.5	26.3	1.33	—	—	—
	8	34.3	34.1	32.9	33.8	2.25	—	—	—
	4	36.4	35.9	36.8	36.4	1.24	—	—	—
	5	38.7	38.1	38.7	38.5	0.91	—	—	—
	3	92.6	93.5	94.3	93.5	0.91	—	—	—
O <sub>2</sub>	8	10.9	11.4	11.1	11.1	2.20	11.5	11.9	11.7
	7	20.4	20.7	20.4	20.5	0.83	21.5	21.0	21.3
	6	38.5	37.6	38.5	38.2	1.36	40.6	40.1	40.4
	9	57.0	55.7	57.0	56.6	1.33	55.9	57.0	56.5
	4	87.3	87.7	87.7	87.6	0.26	92.4	92.4	92.4
	5	92.1	91.9	90.9	91.6	0.71	95.1	95.6	95.4
	3	121	119	120	120.0	0.66	119	120	119.5
	2	140	140	137	139.0	1.15	142	142	142.0
	1	158	156	—	157.0	0.59	159	158	158.5
Total	6	512	509	511	511	0.31	—	—	—
	8	584	584	586	584	0.27	—	—	—
	7	591	602	601	598	1.02	—	—	—
	9	602	591	605	599	1.23	—	—	—
	5	612	611	610	611	0.16	—	—	—
	4	610	611	612	611	0.16	—	—	—
	3	675	677	688	680	1.03	—	—	—
	2	691	703	691	695	0.99	—	—	—
	1	750	752	—	751	0.19	—	—	—

(sum of separately determined carbon dioxide, oxygen and nitrogen tensions) are consistently lower than the values for carbon dioxide and oxygen, because the determination of the large proportion of nitrogen is less influenced by the factors mentioned above; the effect of unwanted gas exchange is negligible in the case of nitrogen and the errors of meniscus readings are relatively very small, leaving variation in the volume of the standard bubble as the principal source of error. The actual values of  $V$  for the nitrogen determinations (not included in the table) range from 0 to 1.28. It should be noted that for partial pressures substantially less than 10 mm. the errors involved in reading the meniscus of the bubble are liable to

increase the overall error of the determination beyond the limits stated above. The values of 1.0 and 1.5 mm. for carbon dioxide in sample 1 represent barely detectable differences between  $V$  and  $V_1$ .

Finally, comparison of tonometric and Winkler determinations of oxygen tension shows a close agreement between the means obtained by the two methods. The maximum difference is one of 5.4% in the case of sample 4, while the mean percentage difference is 3.0. In all but two of the samples, in which the difference between the results of the two methods is insignificant, the Winkler method gives slightly higher results than the tonometric method.

#### SUMMARY

1. A microtonometric method is described whereby tensions of carbon dioxide, oxygen and nitrogen can be determined in fluid samples of 0.3 ml. volume or less, each determination taking 20–25 min.

2. Replicate determinations of the tensions of carbon dioxide, oxygen and nitrogen give maximum coefficients of variability of 3.0, 2.2 and 1.2%, respectively.

3. A comparison of the present method with a micro-Winkler method for the determination of dissolved oxygen shows a close agreement; the mean percentage difference being 3.0.

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# THE RESPIRATORY CENTRE IN THE TENCH (*TINCA TINCA* L.)

## I. THE EFFECTS OF BRAIN TRANSECTION ON RESPIRATION

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### I. INTRODUCTION

Though the concept of a respiratory centre is a very old one, investigations of such a centre, in the vertebrate animals at least, have been confined almost entirely to the mammals. The centre is usually thought of as a group of neurones, situated largely in the bulb, which is responsible for all the respiratory integration. Sensory messages, from stretch and gas-tension receptors, impinge on these neurones and affect the rhythmic activity they produce. These changes in turn, when transmitted to the respiratory effectors via connexions in the cord and the spinal motor neurones, cause modifications of the respiratory movements. The exact anatomical location of such a centre has not been universally agreed on, however, and even its existence has been doubted by some authors. Liljestrand (1953), for example, points out that the possibility of respiratory integration occurring at the spinal level has been almost completely disregarded. In the mammal one of the factors complicating any investigation of the site of respiratory integration is that the major sensory input of the respiratory complex goes into the medulla whilst the motor supply to the respiratory muscles comes from the spinal cord. Consequently, a considerable portion of the central nervous system has to be intact for respiration to continue. In the teleost fish a more compact arrangement appears to exist, with the important respiratory pathways, both afferent and efferent, being carried largely in the Vth and VIIth cranial nerves. It would be interesting, therefore, to see whether integrating neurones exist outside the direct connexions between the sensory and motor nuclei of these cranial nerves.

The technique of making transactions is one of the simplest means of delimiting the respiratory areas of the brain and it has been used a great deal in investigations of the mammalian respiratory centre (Lumsden, 1923; Stella, 1938; Hoff & Breckenridge, 1949). One of the principal limitations of the method is that it is possible for respiratory breakdown to occur when parts of the brain, other than those directly concerned in respiratory integration, are removed. It has been demonstrated that systems existing in the brain stem can affect the performance of quite separately co-ordinated activities, such as the simple reflexes (Magoun,

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1944). A system of this sort, though its removal or damage might cause modification of respiration, would not be considered to be part of a respiratory centre. In spite of this difficulty the transection technique is still a valuable method for delimiting those parts of the brain which can co-ordinate the normal breathing movements.

A few transection experiments have been done on elasmobranchs. Hyde (1904) showed that the respiratory centre in the skate is located in the medulla. She claimed that the centre in these forms is segmentally arranged, the units associated with the sensory and motor areas of the VIIth, IXth and Xth cranial nerves being capable of independent rhythmic activity when separated by transverse cuts. Springer (1928), working on dogfish, was unable to confirm the segmental independence and found that the respiratory region occupied a much greater area of the medulla. No investigations of this sort have been done on teleosts, though it is clear from some of the results of the early workers (see review by Healey, 1957) that damage to the medulla stops the respiratory movements.

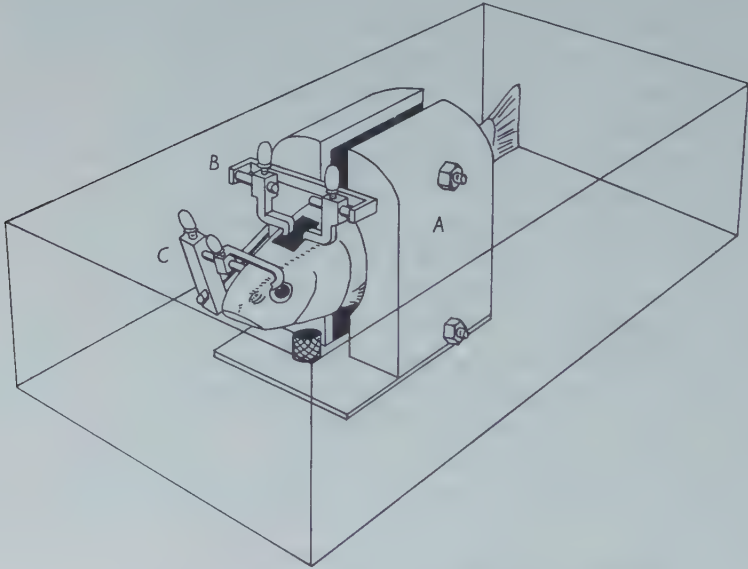


Fig. 1. The clamp used for holding the fish during experiments on the respiratory centre. For further details see text.

## II. METHODS

The experiments were done on fifty-six tench, the majority of which were 15–20 cm. in length and 45–60 g. in weight. In a few later experiments, in which the labyrinth reflexes were tested, slightly larger fish were used because mirrors had to be attached to their eyes. The fish were fixed in a holder (Fig. 1) which has proved to be satisfactory in several different types of experiment on fish respiration. The holder consists basically of three elements, the trunk clamp *A* and the head clamps

*B* and *C*. The trunk clamp is made of wood, grooves being cut out in the two halves to match the body of the fish. One half of the clamp is fixed to the base plate and the other half is adjustable on the two bolts. The head clamps *B* and *C* are retained on the fixed half of the trunk clamp. The clamp *C* consists of two  $\frac{1}{8}$  in. diameter rods in the ends of which are cut V-shaped notches of a size suitable to fit on to the bony supra-orbital ridges of the fish. The rod fixing on to the left supra-orbital ridge is looped over the cranium and slides on the rod of the right side as the diagram shows. This arrangement leaves the left side of the head free from any obstruction which might interfere with recording apparatus. The operative parts of clamp *B* are the two pieces of metal which can slide apart on their supporting rods in much the same way as a surgeon's retractor. In this way the ends of these metal claws can be made to grip the bone of the skull at the edges of the hole made to expose the brain. Both clamps *B* and *C* are independently adjustable so that different sizes of fish can be accommodated in the holder. When all three clamps are used the head of the fish can be rigidly fixed, although the respiratory movements are not interfered with in any way. In many experiments, including most of those described in this paper, it was not necessary to hold the head of the fish quite so firmly and in these cases the clamp *B* was not used.

The holder was fastened to the bottom of a Perspex tank which had a water capacity of 1000 ml., the water being constantly aerated throughout an experiment. All the experiments were done at room temperature (18–20° C.). The movements of the lower jaw and of an opercular flap were recorded on a smoked drum using very light levers so that the movements were not visibly impeded. As far as possible the records were taken before and after the operation to expose the brain, and then after recovery from each transection so that a clear picture of the normal pattern and the subsequent modifications was obtained.

During the operation to expose the brain, and when subsequently the brain transections were being made, the fish were deeply anaesthetized in 0.5–1.0 % urethane solution. They were allowed to recover to a lighter level of anaesthesia (0.2 % urethane approx.) when the recordings of the movements were taken. The transections themselves were made with a cataract knife or with mounted razor blade fragments. A Marconi MME 3 cautery was also used to produce the lesions in some cases, though the results were not noticeably different from those obtained by cutting. After transection there was usually a period of shock when the fish did not breathe and during this period water was passed over the gills from a cannula inserted into the buccal cavity. When respiration had ceased permanently as the result of a brain transection the gills were perfused continuously until the end of the experiment.

### III. RESULTS

#### *A. The effects of brain transections on breathing movements*

The experiments involved transections of the nervous system in both the midbrain and the spinal cord—posterior medulla regions. In the figures which show the results, the brain is drawn as seen from the dorsal surface and the transection levels

are represented by the transverse lines. The order of the lettering in the diagrams represents the sequence in which the sections were made at the various levels. The parts of the brain involved in these vertical transections can be seen in Fig. 2 which shows the brain from a dorso-lateral aspect.

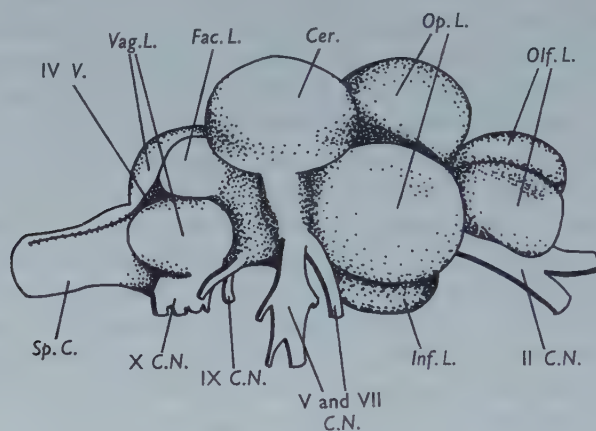


Fig. 2. Tench brain seen from a dorso-lateral aspect. *Sp.C.*, spinal cord; *Vag.L.*, vagal lobes; *Fac.L.*, facial lobe; *Cer.*, cerebellum; *Op.L.*, optic lobes; *Olf.L.*, olfactory lobes; *C.N.*, cranial nerves; *Inf.L.*, inferior lobe; *IV V.*, IVth ventricle.

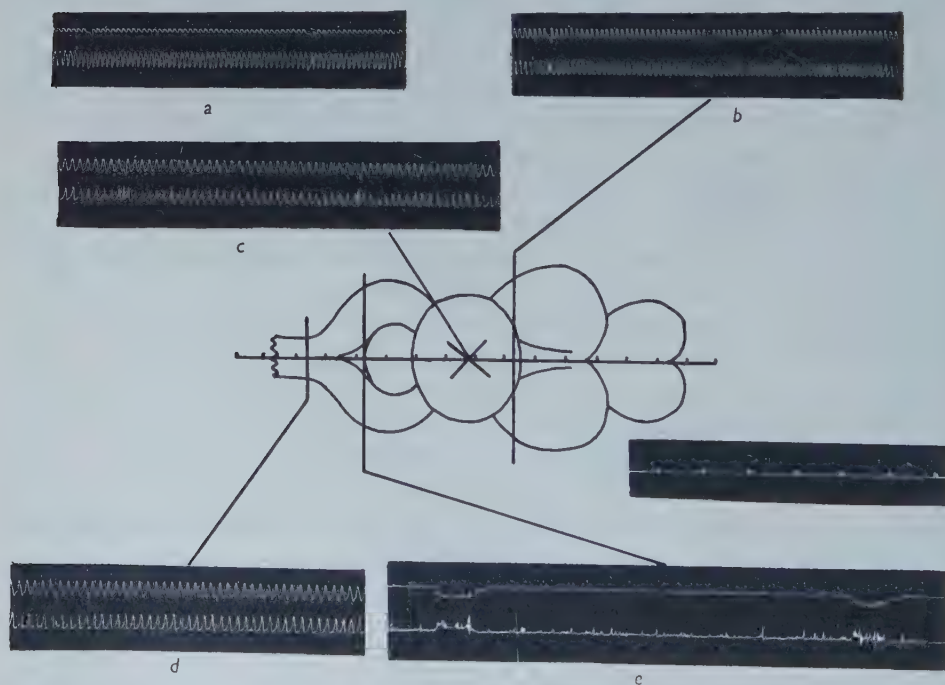


Fig. 3. Respiratory movements before (*a*) and after (*b*, *c*, *d*, *e*) brain transections. Upper trace—movements of operculum (up on trace=operculum closing). Lower trace—movements of mouth (up to trace=mouth opening). Time marker on all figures—10 sec. intervals.

Sections through the optic region lobe, though causing a considerable loss of blood in some cases, had very little effect on the respiratory rhythm after the initial shock period. Certainly the variations were not outside those that normally occurred in the intact animal which had been deeply anaesthetized and then allowed to recover. Transections were made down to the level of the anterior border of the cerebellum, with surprisingly little change in the respiratory pattern (Fig. 3*a, b*). Section at a level lower than this became impossible without damaging the Vth and VIIth cranial nerves, but it was possible to remove the cerebellum completely (Fig. 3*c*) without affecting respiration. Attempts were made to remove, by suction, the more dorsal parts of the medulla beneath the cerebellum and these were always followed by a considerable change in the respiratory pattern. Usually the breathing movements stopped, but on two occasions rhythmic movements were produced even though lesions were made in this way approximately to the level of the Vth motor nuclei.

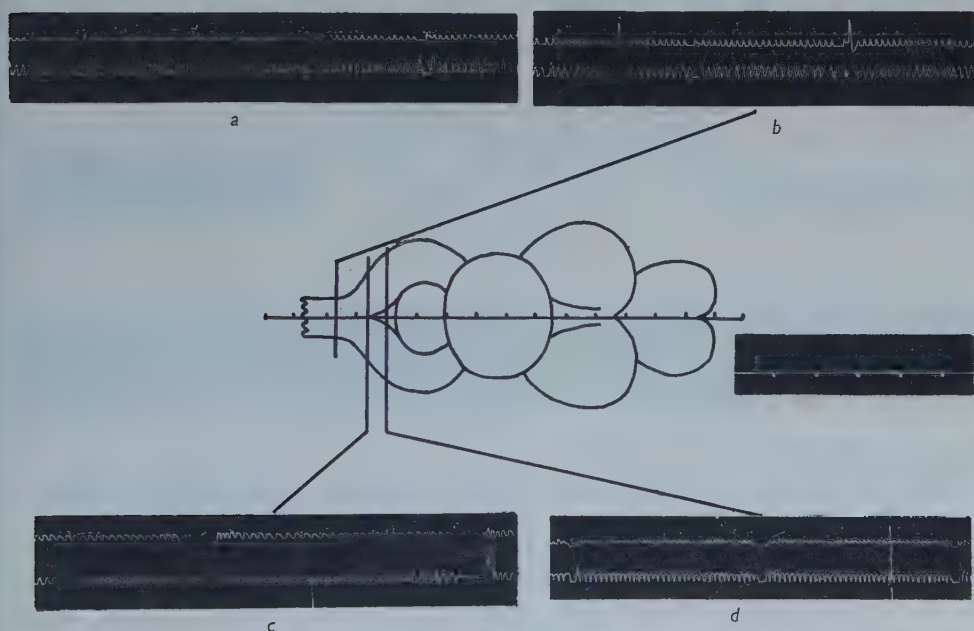


Fig. 4. Respiratory movements before (*a*) and after (*b, c, d*) spinal cord and brain transections. Upper trace—movements of operculum (up on trace=operculum closing). Lower trace—movements of mouth (up on trace=mouth opening).

Transections in the region of the spinal cord and posterior parts of the medulla were more easily performed without causing undue haemorrhage. High sections of the spinal cord in tench never caused respiratory breakdown. In the mammal such sections disrupt important connexions between the medulla and the respiratory motor neurones and the breathing movements cease. In the teleost fish the only connexions which are disrupted by section of the cord or medulla are those to the

spino-occipital efferents and this means that the hypoglossal musculature (m. sternohyoideus in the tench) can no longer function in respiration. This failure had very little effect on the breathing movements as a whole, other muscles being able to participate in opening the mouth (Fig. 4*b*). Transections of the medulla at the level of the obex (Fig. 4*c*) and, further forward, in the middle of the exposed part of the IVth ventricle (Fig. 4*d*) were also possible without affecting the ability of the respiratory complex to produce rhythmic activity. The sections in the region of the IVth ventricle did result in modifications of the breathing rhythm in most cases and

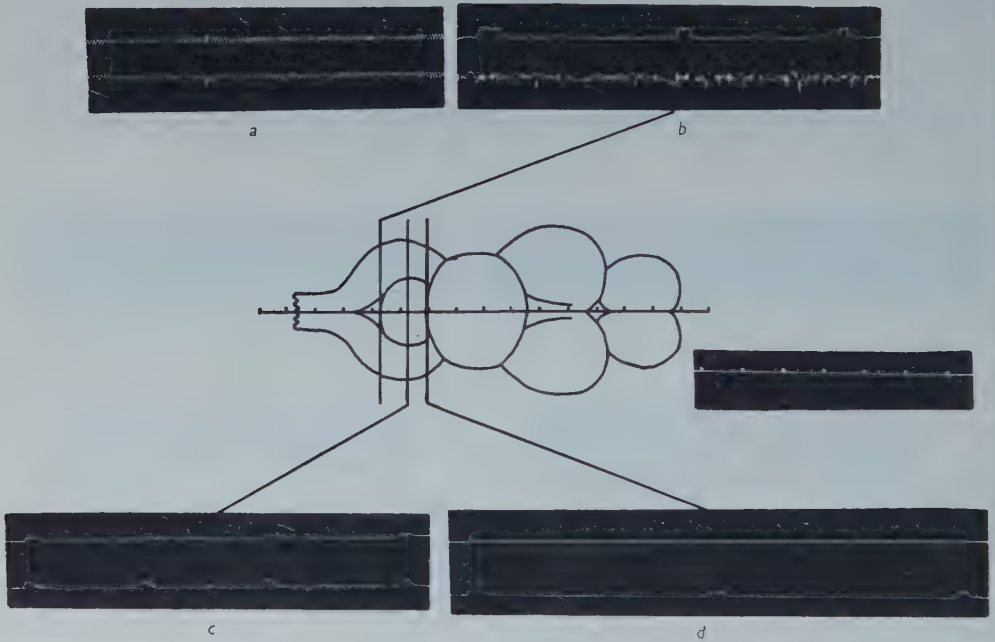


Fig. 5. Respiratory movements before (*a*) and after (*b*, *c*, *d*) brain transections. Upper trace—movements of operculum (up on trace=operculum opening). Lower trace—movements of mouth (up on trace=mouth closing).

in some animals the breathing movements ceased altogether. However, the critical level of transection, after which normal breathing movements ceased in all cases, was at the level of the posterior border of the facial lobe (Fig. 5*b*). After such a section a considerable change occurred in the movement pattern; the usual rhythmic activity disappeared completely and the mouth (and to a lesser extent the operculum) usually made quivering movements. This type of movement continued to some extent with higher sections (Fig. 5*c*), though recovery was often slow and frequently no movements were produced at all.

A very striking feature of the movement pattern, produced after transections had been made in these regions of the posterior medulla, was the appearance of prolonged (5–10 sec.) opercular abductions recurring rhythmically every  $\frac{1}{2}$  to 2 min. (Fig. 4*c*, *d*; Fig. 5*b–d*). The movements of the mouth during these periods of

opercular abduction varied somewhat in different individuals. If rhythmic respiratory movements had not been stopped by the transection then usually the mouth stopped moving in the closed position (Fig. 4*c, d*). If, however, the normal breathing had ceased as a result of a posterior facial lobe transection and the mouth was making quivering movements then there was no change in the mouth movements in some individuals, whilst in others the opercular abduction was accompanied by an increase in the intensity of the mouth quivering. With still higher transection the mouth again closed during the opercular transection (Fig. 5*c*). The slow rhythm persisted even after transection at the posterior border of the cerebellum (Fig. 5*d*).

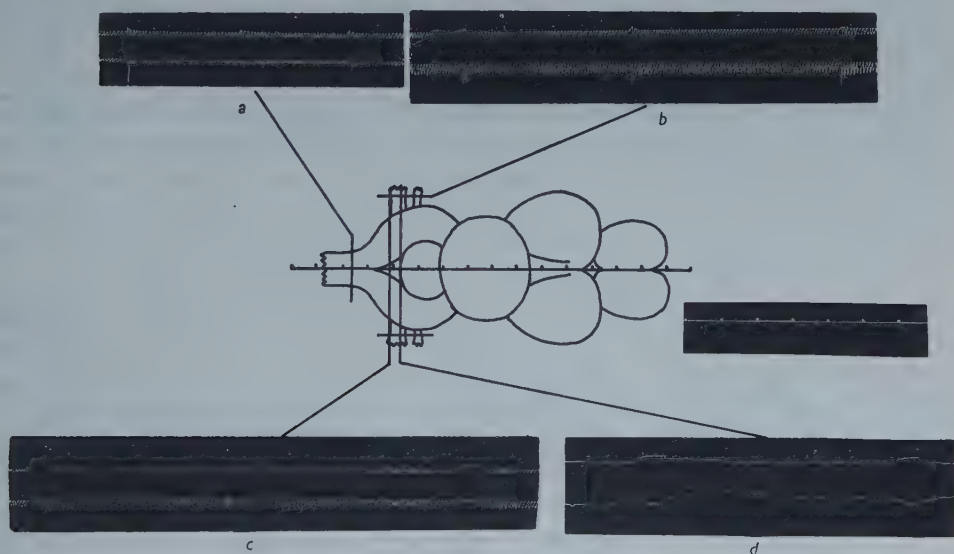


Fig. 6. Respiratory movements after spinal cord (a) IXth and Xth nerves (b), IVth ventricle (c), and facial lobe (d) transections. Upper trace—movements of operculum (up on trace = operculum opening). Lower trace—movements of mouth (up on trace = mouth closing).

Because of their position on the brain stem the IXth and Xth cranial nerves were, of necessity, progressively removed during the hind-brain transections. It was possible therefore that the breakdown of respiration or the appearance of the slow abductions (or possibly both) was due to damage to the nerves rather than to the brain itself. Indeed, Powers & Clark (1942) had concluded that in teleost fish the IXth and Xth cranial nerves, particularly the former, were of fundamental importance in the initiation of the respiratory rhythm. These authors suggested that afferent volleys in the nerves converted tonic activity in the respiratory centre to the normal rhythmic pattern. To decide which of the possible explanations was correct, experiments were done in which the IXth and Xth cranial nerves were sectioned before any brain transections were made. The IXth and Xth nerves were approached at their origin from the brain stem via holes made in the skull in the region of the

labyrinth. The nerve roots were lifted up on glass hooks and then cut with scissors or knife. This ensured that no fragments were left intact. The only effect that sections of these nerves had on the breathing movements was to increase their amplitude; the normal respiratory rhythm continued and the prolonged opercular abductions did not appear (Fig. 6*b*). The amplitude of the movements returned to the normal level over a period of 1–2 hr. Transections at a higher level than the spinal cord did result in the appearance of the abductions (Fig. 6*c*), and even higher transections stopped breathing in the same animal (Fig. 6*d*). It is very likely therefore that the activity seen after transection of the posterior medulla is due to the damage caused to the brain itself and not to the removal of any sensory or motor components carried in the IXth and Xth cranial nerves.

It was of interest to see whether the slow rhythmic abductions of the opercula, like the respiratory movements themselves, were produced by activity in the medulla, or whether they were the result of activity in the higher centres such as the optic tectum. In experiments in which the whole of the fore- and midbrains and the cerebellum were removed it was found that a subsequent transection at the facial lobe level would result in the appearance of the slow rhythm (Fig. 3*e*). The neurones instrumental in the production of this activity must lie in the anterior part of the medulla.

#### *B. The effects of brain transections on a vestibular-eye reflex*

In the mammal it has been shown that certain areas of the brain reticular formation contain neurones having a facilitator or suppressor effect on reflex activity in general (Magoun, 1950). Hoff & Breckenridge (1949, 1954) have proposed that the inspiratory cramps, ensuing after section of the brain at the pontine level, are caused by removal of a generalized suppressor area of the brain stem and are not due to removal of a pneumotaxic centre as proposed by Lumsden (1923) and Pitts (1946). Liljestrand (1953) has extended this concept and suggests that the regions within the medial reticular formation of the bulb, long accepted as the site of the respiratory centre, are themselves generalized facilitator areas. Similarly, the nature of the neural mechanism, which is situated in the facial lobe region of the fish brain and whose removal causes such serious breakdown of the normal breathing rhythm, is not obvious. It may be a vital part of the respiratory centre itself or it may be part of a more generalized system in the reticular formation.

Since any interference with such generalized suppressor or facilitator areas should result in the modification of all reflexes, it should be possible to decide between the possibilities outlined above. The static vestibular-eye reflexes were chosen as being the most suitable for experiments on fish. It must be noted that investigations of the brain-stem reticular formation of the mammal have been largely restricted to examination of its influences on cortical activity or on motor activity from the spinal cord. However, there seems to be no reason to suppose that the eye muscle motor neurones, or the interneurones having synaptic contact with them, are immune from these influences. One reflex at least, the mammalian blink reflex, which is mediated by neurones within the brain, can be suppressed by bulbar stimulation (Magoun, 1944).

In the present experiments the fish was rotated on its long axis from the normal upright position through about  $60^\circ$  (in  $10^\circ$  steps) to a position of right eye down, left eye up. Mirrors were fixed to the left eye and to the body by means of rubber solution and light levers were used to measure both the angle through which the body was rotated and the angle of the eye deflexion. The deflexions were measured before and after the spinal cord and medulla had been transected at various levels, several measurements being made at each level. There was considerable variation between individuals even with intact central nervous systems. One of the six animals tested showed an angular displacement of  $22^\circ \pm 7^\circ$  of the eye relative to the trunk, when the trunk was rotated through  $60^\circ$ . This was the smallest deflexion measured, and, at the other extreme, one fish showed an eye displacement of  $34^\circ \pm 3^\circ$  for the same trunk rotation. After stopping respiratory movements with a transection through the IVth ventricle region, the eye deflexions in each individual were found to be both qualitatively and quantitatively the same as before, provided an adequate supply of water was maintained to the gills. A complicating factor which made the measurement of eye deflexion more difficult after transection at any level was the occurrence of a lot of eye movement particularly in the horizontal plane. The resting position of the eye from which these excursions were made was still obvious and only when it was in this position were the measurements made. Transection caused no enhancement or inhibition of this one reflex which was tested.

#### IV. DISCUSSION

The fact that the slow rhythmic abductions can occur concurrently with a normal respiratory rhythm and can then persist when normal breathing has failed demonstrates that the nervous elements producing the two are largely independent. This slow rhythm cannot therefore be considered as a development of the primary respiratory rhythm as is suggested for gasping respiration in the medullary preparation in the mammal (Brodie & Borison, 1957). It must be an expression either of another pre-existing activity, probably much modified by the effects of transection, or of an entirely new pattern of nervous activity. The evidence favours the former of these two possibilities, since the rhythmically occurring cough is an example of such a slow rhythm in the intact animal and examination does reveal some similarities between the cough and the slow abduction. The frequency range over which the two occur is roughly the same, though the cough in the intact animal is usually more frequent than the slow abduction. Furthermore, after low transections when normal breathing is continued, it is sometimes possible to see transitional states between the normal cough and the prolonged opercular abduction (Fig. 3*a-c*). Finally, it is perhaps significant that, during the initial part of the cough, the operculum opens whilst the mouth closes (Hughes & Shelton, 1958) and the same attitude is usually adopted by these two structures in the prolonged activity after transection. It is suggested, therefore, that the slow abductions represent the activity of neurones situated beneath the cerebellum in the anterior part of the medulla, and concerned in the intact animal with co-ordination of rhythmic

coughing movements. The normal, brief cough is not produced unless the lower levels of the medulla are intact, however, and some part of the integrative mechanism necessary for normal activity must be situated here. Moreover, this posterior part of the mechanism is independent of input from the IXth and Xth cranial nerves and so is entirely central in location (Fig. 6*b*). It is interesting to note in passing that the cough does persist after section of the IXth and Xth cranial nerves, although it is usually thought of as a reflex action initiated by foreign matter on the gills.

Though the transection experiments do not allow an exact anatomical locus to be given to the respiratory neurones, it is clear that these neurones must be contained within the medulla between the transection levels having little or no effect on the respiratory movements. They occur, therefore, below the region where the Vth and VIIth cranial nerves emerge from the brain. A more exact rostral limit to the respiratory neurones cannot be given by this method because these nerves must remain intact for respiration to continue. The caudal limits, on the other hand, can be set more exactly. The experiments on the vestibular-eye reflex show that all reflex activity is not affected by brain transection at the facial lobe level. It is unlikely, therefore, that respiratory failure is due to damage to a generalized suppressor or facilitator area of the medulla. It is also unlikely that this failure is the result of direct injury to the tissue of the brain, causing for example massed injury discharges in neurones unrelated to respiration. Similar injury effects must have been caused by the more posterior brain sections, some of which were very near the critical level, and yet these had no fundamental effect on the breathing rhythm. Application of procaine to the cut surface of a brain, transected at the facial lobe level, had no effect on the random quivering movements of the mouth and opercula until it was present in sufficient concentration to act as a general anaesthetic, when all movements stopped. Furthermore, no indication of the normal rhythm was ever seen, though a fish was kept alive up to 3 hr. after facial lobe transections. During this time an effect due to an injury discharge should have disappeared.

In this case, therefore, respiratory failure after brain transection at the posterior border of the facial lobe is apparently due to removal of part of the system directly involved in respiratory integration. There is very little interference with the sensory or motor pathways of the Vth and VIIth cranial nerves as the nuclei of these nerves are situated in the anterior part of the medulla. The descending ramus of the Vth cranial nerve, and a sensory ramus of the VIIth cranial nerve ending in the large facial lobe, are the only components extending back into the region of the medulla involved in these transections. The descending ramus of the Vth runs back to a secondary gustatory nucleus and is sectioned at lower levels than those stopping the breathing movements. It can also be shown that transections causing respiratory failure need not involve the facial lobe. Furthermore, the respiratory neurones or connexions situated at this critical level are not part of an essential reflex involving the IXth and Xth cranial nerves, as the experiments have shown. Therefore, as direct sensory and motor pathways are not involved, transection in the facial lobe region removes neurones which are part of an intermediate integrating system between the sensory and motor nuclei of the cranial nerves involved in respiration.

These neurones are essential in the production of the normal respiratory rhythm and so are part of what could properly be called a respiratory centre. It is unlikely that the whole of such a centre is removed by transection at the facial lobe level. The centre probably consists of a large number of interacting neurones and removal of a relatively small number of these would be sufficient to cause respiratory failure. The work on mammals would suggest that the neurones are situated in the reticular formation of the medulla. However, the site of the neurones and their extension within the region of the medulla delimited by the transections are problems which can be solved only by the use of other techniques.

## SUMMARY

1. The effects of brain transections on the breathing movements of the tench are described.
2. The whole of the mid- and forebrain, and the cerebellum, can be removed without producing any change in the breathing movements.
3. Normal movements continue after section of the IXth and Xth cranial nerves.
4. Transections of the spinal cord and posterior medulla are without effect on the breathing rhythm until they reach a level just behind the facial lobe. The breakdown of respiration produced by transection at this level is interpreted as being due to removal of part of the respiratory centre.
5. Rhythmically repeated movements in which the opercula abduct and the mouth closes are seen after transection in the posterior parts of the medulla. These movements are thought to be due to activity in neurones which are responsible for co-ordination of the coughs in the intact animal. These neurones are situated in the anterior part of the medulla, beneath the cerebellum.

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# THE EFFECTS OF HIGH TEMPERATURES ON ROACH (*RUTILUS RUTILUS*)

## I. THE EFFECTS OF CONSTANT HIGH TEMPERATURES

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### INTRODUCTION

The thermal relations of fishes have been much investigated in Canada and terms describing various states have been defined by Fry and his colleagues from their own observations (Brett, 1941, 1944, 1946; Fry, Brett & Clawson, 1942; Fry, Hart & Walker, 1946; Fry, 1947; Hart, 1947, 1952; Black, 1953) and from the earlier work of Hathaway (1927) and Loeb & Wasteneys (1912).

The temperature relations of British fishes are not very well understood and the available information mainly concerns growth and development (Audigé, 1921; Gray, 1928; Wood, 1932; Brown, 1946, 1951). Cocking (1957), however, showed that the temperature range for good health in the roach is curtailed 4 or 5° C. below the ultimate upper lethal temperature for the species.

This study was designed to provide reliable figures for the upper lethal temperatures and the thermal tolerance for a common British fish. The roach was chosen because it is a 'coarse' fish that is very common in the lowland reaches of rivers where pollution is most frequent and because Hartley (1947) had investigated its natural history. Particular attention was paid to its behaviour because although death has often been used as the criterion of the effectiveness of pollution (Fry *et al.* 1946; Wuhrmann, 1952; Downing, 1954), nobody has described the behaviour of the dying fish or tried to relate this to the lethal factors involved. Wells (1914) and Rubin (1935) both reported that fish become more active at high temperatures and this must affect the fish's chance of survival.

Winterstein (1908) reported that the asphyxial concentration of oxygen of 'Rutilus' at room temperature was 0.4 ml./l., but it is not clear whether these fish were roach or rudd. The ability to withstand low concentrations of oxygen at high temperatures must be of survival value in polluted habitats, so this investigation included estimations of the asphyxial tensions of oxygen of the roach at 30 and 32° C.

Poor condition in the experimental fish is often an important unknown factor in fish physiology. Difficulty in keeping fish healthy (Wells, 1935; Job, 1955) may lead to difficulty in acclimatizing them properly (Hart, 1952). The aim of keeping the fish in first-class condition for the experiments and keeping them in the laboratory at known temperatures was realized; the temperature history of some of the fish used in the tests was known to within  $\pm 0.1^\circ$  C. for more than 3 months.

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*Definition of terms*

The terms used are those defined by Fry *et al.* 1946.

Below and above *the acclimatization temperature*, i.e. the temperature at which the fish have been kept for at least a week, is *the zone of tolerance*, which contains all temperatures at which over 50% of the fish can live indefinitely. The zone of tolerance is bounded by *upper and lower incipient lethal temperatures*, which are the temperatures at which 50% of the fish die within a week. Above the upper incipient and below the lower incipient lethal temperatures are *zones of resistance*, which include all temperatures at which more than 50% of the fish will die. The time taken to die decreases as the difference between the acclimatization temperature and the temperature to which the fish are transferred increases. The temperature in the zone of resistance at which all the fish die within 10 min. is *the temperature of instantaneous death*. Raising the acclimatization temperature raises the upper incipient lethal temperature until the *ultimate upper lethal temperature* for the species is reached, i.e. the highest upper incipient lethal temperature that cannot be increased by raising the acclimatization temperature. Similarly, lowering the acclimatization temperature lowers the lower incipient lethal temperature until *the ultimate lower lethal temperature* is reached.

## MATERIALS

The tests were done in a tank room designed to provide constant environmental conditions for growing or acclimatizing fish. Brown (1951) has described an earlier model which resembles the present one in all essentials.

Table 1. *The average concentrations of ions in Strand, London, tap water. (Figures supplied by the Metropolitan Water Board)*

Chemical composition as p.p.m.			
Calcium (as Ca)	97	Sulphates (as SO <sub>4</sub> )	49
Magnesium (as Mg)	4	Nitrates (as NO <sub>3</sub> )	15
Sodium (as Na)	20	Silicates (as SiO <sub>2</sub> )	10
Potassium (as K)	5	Alkalinity (as CaCO <sub>3</sub> )	243
Chlorides (as Cl)	27	Total dissolved solids	380

Strand (London) tap water is very hard and contains unusually high concentrations of chloride, sulphate and nitrate ions (Table 1). From November to May it passed through a charcoal filter before entering the tanks to remove possible traces of free chlorine and chloramines. The tanks received 12 hr. of light daily from 20 W., fluorescent, 'Daylight' tubes. During the other 12 hr. each day the tanks were in complete darkness.

Most of the roach used were small (2–15 g.) and were caught by seine in Tarn Hows in Furness, Lancashire. Some larger fish (20–40 g.) used in earlier tests came from ponds and rivers in Essex and Cambridgeshire and from a trout stream in Hertfordshire. The fish were, therefore, from natural populations and probably

of various ages. The effect of transfer from soft Tarn Hows water (total dissolved solids about 40 p.p.m.) to hard Strand tap water (total dissolved solids about 380 p.p.m.) may have produced a slight increase in resistance to high temperatures (Halsbrand, 1953) but this was not investigated.

A week after arrival, when the fish were feeding, the temperature was raised to the acclimatization temperature at a rate not exceeding  $1^{\circ}\text{C}$ . a day and the fish were acclimatized for as long as possible. The fish were kept at  $20^{\circ}\text{C}$ . as a basic acclimatization temperature and were then held for at least a week at the experimental acclimatization temperature. The fish were given as much minced liver as they would eat five or six times a week and lettuce leaves three or four times a week.

#### METHODS

I investigated the survival times at constant lethal temperature by the method used by Fry *et al.* (1946) to investigate the thermal relations of *Salvelinus fontinalis*.

Roach were acclimatized to  $17$ ,  $20$ ,  $23$ ,  $26$  and  $30^{\circ}\text{C}$ . for periods up to 3 months and then moved quickly to higher lethal temperatures. The jump in temperature was instantaneous to avoid further acclimatization and the tests were carried on for up to 36 hr. Fish were also acclimatized to  $32$  and  $33^{\circ}\text{C}$ . but they lost condition rapidly at these temperatures which were maintained for only a few days. When tested at higher temperatures, these fish took up to 4 days to die and, as it was impossible to maintain constant watch over this period of time, no median survival times could be calculated, though the upper incipient lethal temperature was estimated in each case. The fish were starved for 24 hr. before being caught and put into the test tank. Eight fish were used in each test except when acclimatized to  $30$ ,  $32$  and  $33^{\circ}\text{C}$ . when only five fish were used. The test tank was a standard 50 l. tank, stirred and aerated by compressed air, with water flowing through and the temperature controlled to within  $\pm 0.1^{\circ}\text{C}$ .

The criterion of death was that opercular movements, which persisted after the fish was unable to swim, had stopped; if the fish were removed before this stage, it would often recover. Sumner & Doudoroff (1938) using the cessation of respiratory movements as the criterion of death in *Gillichthys*, found that this fish sometimes recovered when placed in cooler water. In the present tests only one out of all the fish tested recovered when placed in cooler water, so the determination of the death point was an accurate or conservative estimate.

The time of death of each fish was recorded and the fish was then weighed, measured from the tip of the snout to the fork of the tail and opened to determine sex, to see if the heart was still beating and to examine the other organs. Notes were kept of the behaviour of the fish and in many cases individuals were watched continuously from being put into the tank to dying.

The asphyxial concentration of oxygen at  $30$  and  $32^{\circ}\text{C}$ . was measured by sealing a roach in a container and estimating the oxygen concentration when it rolled over. It was starved for 48 hr. and put into a light-proof water-bath at the experimental

temperature. Water, saturated with oxygen at the experimental temperature, flowed through the container overnight to allow the fish to recover from catching and handling before the flow was stopped. The dissolved oxygen and total ammonia concentrations, alkalinity and pH were measured just before stopping the flow through the container and immediately after the fish had rolled over, and from these measurements the dissolved oxygen, free ammonia and carbon dioxide concentrations could be calculated. The asphyxiated fish was weighed and put into well-aerated water to see if it would recover.

Throughout all experiments temperature was measured to the nearest  $0.1^{\circ}\text{C}$ . Oxygen was estimated by unmodified Winkler's technique. At high temperatures, in spite of tight fitting stoppers, small bubbles of air (about  $0.1\text{ ml.}$ ) were drawn in as the sample cooled. This was prevented by submerging the bottles in boiled-out water; the small volume of water drawn in contained a negligible quantity of oxygen. Carbon dioxide was estimated graphically (Moore, 1939) from measurements of pH, alkalinity and temperature. Ammonia was estimated photo-electrically using Nessler's reagent and a Hilger 'Spekker'. Only free ammonia affects fish (Wuhrmann, 1952) and the concentration was calculated from the total ammonia, pH, temperature and total dissolved solids (Downing, personal communication). Alkalinity (equivalent to carbonate hardness) was measured by titrating  $0.1\text{N}$ -hydrochloric acid against  $25\text{ ml.}$  of water, using B.D.H. '4.5' indicator. Total dissolved solids were estimated as recommended by Thresh, Beale & Suckling (1949). The pH was measured on a Cambridge bench-type pH meter. The instrument was compensated for temperature and the buffers used for calibration were kept at the temperature of the water samples.

## RESULTS

### *Survival at constant high temperatures*

Table 2 shows the mean survival time for roach acclimatized at  $17$ ,  $20$ ,  $23$ ,  $26$  and  $30^{\circ}\text{C}$ . and tested at higher, lethal temperatures. Following Fry *et al.* (1946), the geometric mean time has been used. In Fig. 1, test temperature is plotted against time, on a semi-log scale. For any acclimatization temperature, the survival time decreases as the test temperature increases and the resistance to any given lethal temperature increases with a rise in acclimatization temperature. The graph makes it possible to estimate the median survival time for a group of fish acclimatized to any of the temperatures shown and tested at a higher temperature. The lines representing tests with fish acclimatized to  $26$  and  $30^{\circ}\text{C}$ . terminate at vertical lines to the right; beyond these less than  $50\%$  of the fish would die. There must be corresponding points on the  $23$ ,  $20$  and  $17^{\circ}\text{C}$ . lines but they are off the graph, to the right.

Figure 2 summarizes the information in Table 2 and Fig. 1 and also includes the upper incipient lethal temperatures for fish acclimatized to  $32$  and  $33^{\circ}\text{C}$ . It shows, for each acclimatization temperature, the size of the upper part of the zone of tolerance, the upper incipient lethal temperature, the zone of resistance and the

Table 2. The geometric mean survival time for samples of roach acclimatized at a given temperature and tested at higher, lethal temperatures

Test temp. (° C.)	Survival time (min.) for acclimatization temp.				
	17	20	23	26	30
33.5	—	—	—	—	100
33.0	—	—	—	—	218
32.5	—	—	—	57	248
32.3	—	—	—	106	—
32.1	—	—	95	131	—
31.9	—	—	123	—	—
31.7	—	—	—	256	—
31.6	—	—	226	—	—
31.5	—	0	—	—	—
31.2	—	—	295	—	—
31.0	—	87	—	—	—
30.9	—	170	—	—	—
30.8	—	244	—	—	—
30.1	—	675	—	—	—
29.9	34	—	—	—	—
29.2	327	—	—	—	—

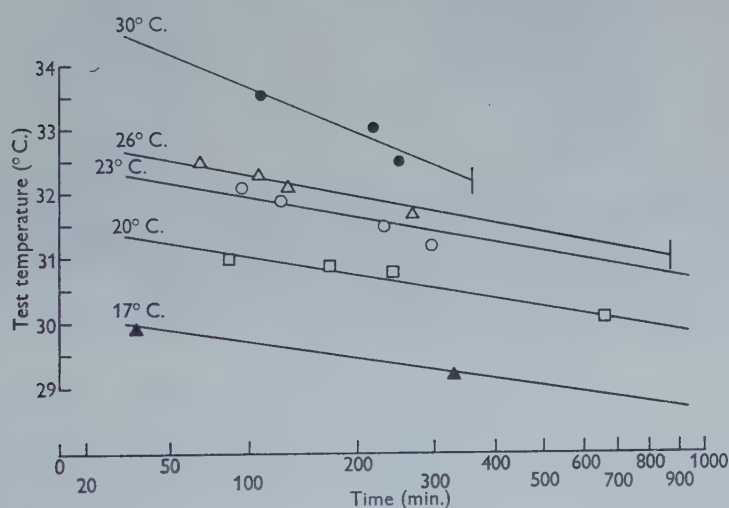


Fig. 1. Median survival time of roach at constant lethal temperatures when acclimatized to 17, 20, 23, 26 and 30° C. Plotted on semi-log scale. Drawn from data in Table 1. Figures above lines—acclimatization temperature. To the right of the vertical lines less than 50 % of a sample would die.

temperature for instantaneous death which marks the upper limit of the zone of resistance.

Thus for roach the ultimate upper lethal temperature is approximately 33.5° C. As the acclimatization temperature is lowered, the upper incipient lethal temperature is lowered and the zone of tolerance is, therefore, larger at lower acclimatization temperatures.

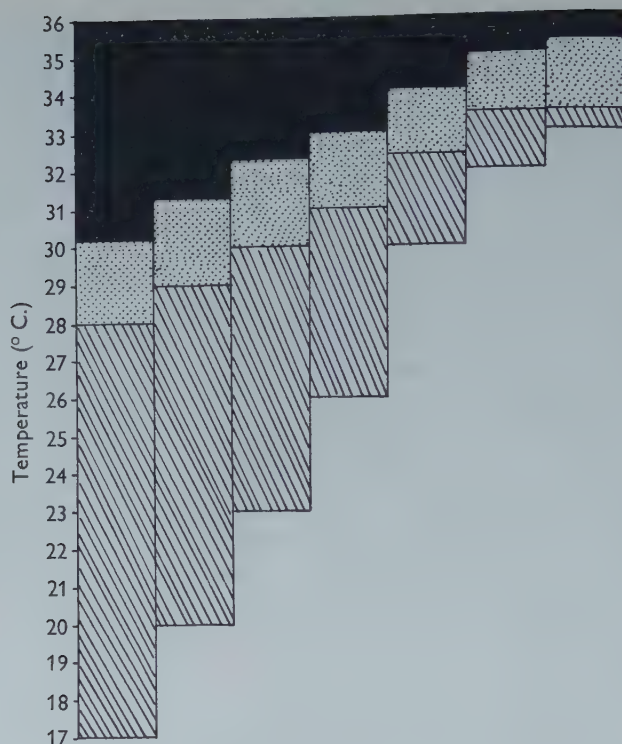


Fig. 2. Diagram showing the thermal relations of roach when acclimatized to 17, 20, 23, 26, 30, 32 and 33° C. Cross-hatched—zone of tolerance; dotted—zone of resistance; black—temperatures at which death is instantaneous. The zone of resistance is limited below by a horizontal line representing the upper incipient lethal temperature and above by a line representing the temperature for instantaneous death. The lower horizontal line limiting the zone of tolerance represents the acclimatization temperature.

#### *Thermal tolerance*

Figure 3 shows the thermal tolerance of the roach in the way originally adopted by Fry *et al.* (1942) for year-old goldfish. The lower incipient lethal temperatures for roach acclimatized to 30 and 23° C. were estimated so that the thermal tolerance could be calculated.

Upper incipient lethal temperatures for fish acclimatized to 17, 20, 23, 26, 30, 32 and 33° C. are plotted to show the upper lethal limits; the line joining these points is produced to the left to meet the axis, since no upper incipient lethal temperatures were measured for acclimatization temperatures below 17° C. An increase in acclimatization temperature above 32° C. does not raise the upper incipient lethal temperature so the line joining the upper incipient lethal temperatures becomes horizontal and cuts the line drawn at 45 degrees to the axes at 33.5° C. From the intersection a perpendicular is dropped to cut the lower lethal line which is drawn through the lower incipient lethal temperatures for roach acclimatized to 30 and 23° C. For a species with an ultimate lower lethal temperature

above the freezing point of water, the lower lethal line would become horizontal and cut the 45-degree line to the left, but it is probable that the ultimate lower lethal temperature of the roach is about  $0^{\circ}\text{C}$ . and this has been assumed in constructing the diagram. The area contained by the two axes, the upper and lower lethal lines and the perpendicular therefore represents all the thermal variations that roach can tolerate indefinitely. This area equals the thermal tolerance of roach and is 770 square units. The upper incipient lethal temperature of roach increases by about  $1^{\circ}\text{C}$ . for each  $3^{\circ}\text{C}$ . rise in acclimatization temperature. Brett (1944) found a similar relationship between upper incipient lethal temperature and acclimatization temperature in the goldfish and *Ameiurus*.

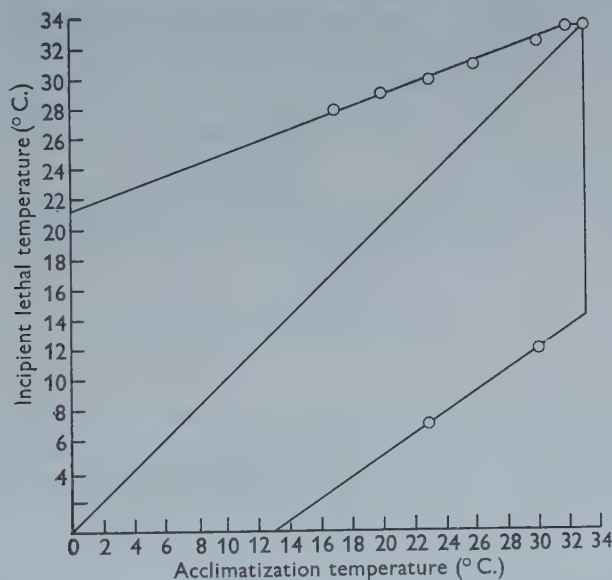


Fig. 3. The thermal tolerance of roach (770 units).

### Behaviour

Roach living in acclimatization tanks at  $20^{\circ}\text{C}$ . were light, olive green in colour and their breathing was slow and discontinuous. They always formed a shoal at the end of the tank away from the observer a few inches off the bottom; even three or four roach would form a shoal and show no sign of individual territories. They only moved into the upper and middle parts of the tank when fed; when transferred from one tank to another they swam to the bottom and remained still or swam quietly, though for the first few hours after transfer they were easily disturbed.

The behaviour of roach when put into lethal temperatures (Table 3) can be divided into five stages: initial distress, recovery, final distress, loss of control and death. Some of the fish in a test went through the stages more quickly than others and at any moment the tank would contain fish in various stages of distress, recovery and death.

Table 3. *Behaviour of roach when transferred to constant higher temperatures*

Variation 1: Characteristic of fish acclimatized to 23° C. and above.

Variation 2: Characteristic of fish put into temperatures above the temperature for instantaneous death.

Variation 3: Characteristic of fish tested close to the upper incipient lethal temperature.

Stage		Symptoms	
Period of no response			
Initial distress	Variation 2	In upper part of water; rapid glides hitting obstacles; cutting surface; rolling and pitching; breathing erratic	Variation 1
Recovery		Move to bottom; swim more steadily; form shoal; pass air bubbles; defaecate; rapid breathing	
Final distress		Black pattern; leave shoal; move up and down tank; lose depth control when still; bursts of weak jerky swimming towards end	Variation 3
Loss of control		Float on side or back; body stiff but eyes and pectoral fins move; breathing very rapid, shallow and continuous	
Death		Motionless (opercular movements are last to stop)	
Complete recovery		Breathing and colour normal	

When a fish acclimatized to 17° C. was put into a test tank at 29° C. it showed no response for the first  $\frac{1}{2}$ –2 min. and then entered *the stage of initial distress*, characterized by almost complete loss of control. When initial distress was acute, the fish made erratic glides at a speed that the eye could not follow and banged against the thermostat, heating tube and sides of the tank. The fish moved in the upper layers of water and frequently cut the surface; occasionally, fish leaped half out of the water and on two occasions jumped clear of the tank. Breathing was erratic and the fish rolled, pitched and swam vertically up and down the tank. In a less acute form, distress was shown by bursts of very rapid swimming which alternated with quiet pauses. Initial distress usually lasted about 15 min. and was followed by *the stage of recovery*. The fish moved towards the bottom of the tank and began to pass bubbles of air from the mouth; it sometimes rose slightly in the water to do this but usually remained near the bottom. Swimming became steadier and the fish began to form a shoal. Very rapid breathing (about 200 movements a minute) and frequent defaecation were also characteristic. The recovery stage sometimes lasted for several hours but it was succeeded by *the stage of final distress*, which was marked by the development of a characteristic black pattern on the anterior, lateral and dorsal surfaces of the fish (Fig. 4). The black pattern contrasted strongly with the pale olive green of the rest of the body and it increased in intensity up to the end of the stage of final distress, but became fainter again as the fish died. As each fish became distressed, it left the shoal and began moving continually from the bottom of the

tank to the top and back to the bottom again. Some fish showed difficulty in maintaining their position in the water by floating upwards when not actively swimming and some passed bubbles of air again, though usually at, or near, the surface of the water. The fish passed gradually from this stage to *loss of control*, i.e. when they rolled over and could no longer right themselves even if stimulated. Loss of control began as the swimming muscles passed into rigor, resulting in weak, jerky swimming movements with frequent half-rolls. Fish frequently alternated between lying on their sides or on their backs and swimming unsteadily round the tank. When unable to swim they made feeble body movements, but eventually they became unable to move the body at all, even when tapped with a glass rod, though the mouth, opercula, pectoral fins and eyes continued to move. Occasionally the fish would give a violent coughing reflex when the opercula were widely distended, but the last visible movement before *death* was very shallow breathing. The fish, therefore, died from the posterior end forwards.

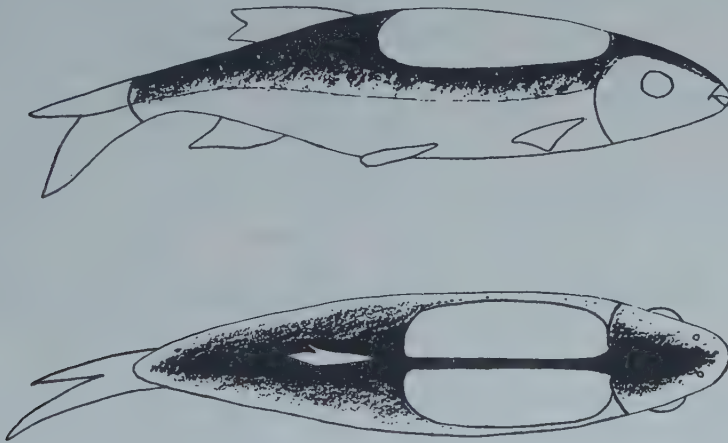


Fig. 4. Diagram showing the lateral and dorsal views of the characteristic black pattern which appeared in the stage of final distress in fish acclimatized to 20° C. and above.

There are three main variations on the scheme outlined above. (1) Fish acclimatized to 20° C. and tested 11 or 12° C. higher show only slight initial distress and this is further reduced in fish acclimatized to 23 and 26° C. until for those acclimatized at 30° C. there is no initial distress and the fish pass straight into the later part of the recovery phase, the first signs of final distress being the appearance of the black pattern. (2) When the jump in temperature takes the fish above the temperature for instantaneous death, it is impossible to distinguish between separate stages, since the fish die in less than 5 min. and the behaviour resembles a short period of initial distress followed by death. (3) At temperatures close to the upper incipient lethal temperature, the fish enter a reduced stage of initial distress followed by the stage of recovery with most of the characteristic signs, including darkening and rapid breathing. Some of the fish become finally distressed and die but others recover completely, darkening disappears and breathing returns to normal. It is

often possible to tell which fish will die and which will recover since if the characteristic black pattern appeared (Fig. 4) the fish invariably died, but uniform darkening usually meant that the fish would eventually recover.

When the fish had died, they were opened. In almost every case the heart was still beating and although in some cases the beat was weak and irregular it was often strong and regular. The gall bladder was abnormal, however; in normal roach it is oval and clear light green in colour whereas in test fish it was frequently cloudy and dark green. In fish with long exposures to high temperatures it was black and very large. The black colour was caused by the concentration of bile in the bladder, since when the bile was examined in a strong light in a fine pipette it was clear but a very dark green.

Table 4. *The concentrations of dissolved oxygen, free carbon dioxide and free ammonia as parts per million, when roach asphyxiate themselves at 30 and 32° C.*

	Dissolved oxygen as p.p.m.	Free carbon dioxide as p.p.m.	Free ammonia as p.p.m.
Fish asphyxiated at 30° C.	0.80	10	0.02
	1.00	10	0.01
	1.00	6	0.07
	0.70	6	0.03
	0.60	3	0.05
Fish asphyxiated at 32° C.	0.84	6	0.02
	0.96	7	0.02
	0.68	5	0.01
	0.80	4	0.03
	0.66	7	0.01

#### *The asphyxial concentration of oxygen*

Table 4 shows the concentrations of oxygen, free ammonia and free carbon dioxide at the time of asphyxiation for roach at 30 and 32° C.

At 30° C. the range of asphyxial concentrations of oxygen is 0.60–1.00 mg./l. (mean 0.82 mg./l.) and at 32° C. it is 0.66–0.96 mg./l. (mean 0.79 mg./l.). The ranges and the means are, therefore, very similar. At 32° C. none of the fish recovered when transferred to well-aerated water, but at 30° C. three out of five recovered.

#### DISCUSSION

Young goldfish, *Carassius auratus* (Fry *et al.* 1942), are the most eurythermal fish known (upper ultimate lethal temperature 40.0° C., thermal tolerance 1220 units) and chum salmon, *Oncorhynchus keta* (Brett, 1952), the most stenothermal fish known (upper ultimate lethal temperature 23.9° C., thermal tolerance 468 units). The ultimate upper lethal temperature of the roach is about 33.5° C. (Fig. 2) and the thermal tolerance 770 units (Fig. 3). It is, therefore, about halfway between the most eurythermal and stenothermal fish known and has similar thermal limits to *Perca flavescens*, the yellow perch (Hart, 1952; Fry, 1957; upper ultimate lethal temperature 33.0° C., thermal tolerance 742 units).

It is difficult to compare the thermal relations of roach with those of other British fish because the latter have not been measured. It is clearly more eurythermal than brown trout, *Salmo trutta*, which will not grow above 21° C. (Brown, 1946), which probably have an upper lethal temperature of about 25° C. and which have a high mortality rate when reared from the egg at 15° C. (Gray, 1928). Brook trout, *Salvelinus fontinalis*, have an ultimate upper lethal temperature of 25.3° C. and a thermal tolerance of 625 units (for Canadian fish) and are probably more stenothermal than *Salmo trutta*. The roach is probably more eurythermal than the minnow, *Phoxinus phoxinus*. Fortune (1955) reported that minnows died in tap water at temperatures above 23° C. but this observation needs verification, since Barrington & Matty (1954) kept *Phoxinus* at 26° C. and the ultimate upper lethal temperature is probably about 30° C. Since *Perca flavescens* and *P. fluviatilis* are closely allied it is possible that the British perch and roach have similar thermal limits.

The results of the temperature-time experiments (Fig. 2) lead to two conclusions of importance in nature. First, fish will not be killed by a sudden rise in temperature, providing the increase does not take the fish out of the zone of tolerance for the particular acclimatization temperature. Roach acclimatized to 17° C. can withstand an instantaneous jump of 10° C. without dying and they will be able to survive an even greater jump from water colder than 17° C., since the size of the zone of tolerance increases with decrease in acclimatization temperature (Fig. 2). If the relationship between increase in acclimatization and upper incipient lethal temperatures (1° C. rise in upper incipient lethal temperature for each 3° C. rise in acclimatization temperature) holds over the whole thermal range of the species, the zone of tolerance should increase by about 2° C. for each 3° C. fall in acclimatization temperature. Secondly, even if the temperature rises instantaneously to a lethal level (i.e. in the zone of resistance) the fish will survive for a length of time that is a function of the increase in temperature. Roach acclimatized to 20° C. can survive water at 31° C. for a median time of 87 min. and this would give them time in nature to move to cooler water or would allow them to survive if the increase in temperature were only temporary. Under natural conditions, therefore, roach should be most susceptible to a sharp rise in temperature when they are already living near their ultimate upper lethal temperature, but it is difficult to correlate fish kills in nature with temperature, because temperature effects are complicated by the effects of temperature-dependent factors. These results do not mean, however, that fully acclimatized roach can live indefinitely at high sublethal temperatures. When kept at 30° C. for long periods, roach lose condition rapidly and die (Cocking, 1957). Long exposures at temperatures 3–4° C. below the ultimate upper lethal temperatures are, therefore, harmful and result in a loss of condition which would damage a natural population.

The behaviour of roach in constant lethal temperatures depends on the size of the jump in temperature to which the fish are subjected. At temperatures near the upper incipient lethal temperature roach show distress but subsequently recover. At higher temperatures the fish are unable to adjust themselves and die in a

characteristic manner, i.e. from the posterior end forwards. Sumner & Doudoroff (1938) noted that *Gillichthys* died in a similar manner at high temperatures and in potassium cyanide solutions, boiled sea water and anaesthetics. The hearts of the roach were still beating when the body cavities were opened after death. The work of Vernon (1899) and Battle (1926) suggests that heart muscle is more sensitive to heat than skeletal muscle. Since the heart beat was sometimes abnormal, however, it is possible that the heart had stopped and it started to beat again when the fish cooled on being taken out of water and the body cavity opened.

The asphyxial concentrations of oxygen of roach (Table 4) are some of the lowest recorded for any species and confirm Winterstein's (1908) results. A low asphyxial tension implies that a species must have a blood of low loading tension and it is possible that the roach, like the carp (Nicloux, 1923), uses its haemoglobin only when in water of very low oxygen concentrations or when very active. The asphyxial concentrations at 30 and 32° C. apply only to fish acclimatized to those temperatures. Aitken (personal communication) has shown that the oxygen consumption of roach muscles from fish acclimatized to 10° C. was much higher at 20° C. than the oxygen consumption of muscles from fish acclimatized to 20° C. Wells (1935) also showed that acclimatization to high temperatures involves a proportionate reduction in metabolic rate. Since the asphyxial concentration of fish depends, in part, on the metabolic rate, the asphyxial concentration of roach at 30° C. following a sudden rise in temperature would be higher than 0.8 mg./l., the amount measured for roach acclimatized to that temperature.

#### SUMMARY

1. The temperature at which 50 % of a sample of roach (*Rutilus rutilus*) die within a week cannot be raised above 33.5° C. by raising the acclimatization temperature.
2. The roach is about as eurythermal as the yellow perch (*Perca flavescens*).
3. The mean asphyxial concentration of oxygen at 30 and 32° C. is approximately 0.8 mg./l.
4. Median survival time at any lethal temperature increases with increase in acclimatization temperature; survival time for any acclimatization temperature decreases as test temperature increases; the temperature at which 50 % of a sample die within a week rises by about 1° C. for each 3° C. rise in acclimatization temperature.
5. The behaviour, on transfer to higher temperatures, depends on the acclimatization temperature and the size of the jump in temperature and can be divided into five characteristic stages.
6. Dying fish develop a black pattern; myotomic swimming muscles die first and opercular muscles last. The heart was still beating when the fish were opened but the gall bladder was abnormal.

This work was carried out while holding a British Celanese Studentship. I should like to thank Mrs G. C. Varley (Dr M. E. Brown) for her advice and encouragement throughout.

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# THE EFFECTS OF HIGH TEMPERATURES ON ROACH (*RUTILUS RUTILUS*)

## II. THE EFFECTS OF TEMPERATURE INCREASING AT A KNOWN CONSTANT RATE

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### INTRODUCTION

The investigation of thermal relations of aquatic organisms by continuously raising the temperature at a known rate has been criticized because this method involves two variables, time and temperature. In spite of this, however, the method can provide useful information about the speed of acclimatization, the behaviour of fish in constantly changing temperatures and the effects of different rates of temperature rise on certain physiological processes, e.g. the excretion of ammonia. Criticism was particularly pertinent when the methods of raising the temperature were crude and the rate of temperature rise not constant throughout an experiment (Vernon, 1899; Huntsman & Sparks, 1924). In this work, however, a modified thermostat allowed the temperature of the water to rise at known constant rates of from  $1/20$  to  $8/10^{\circ}$  C. an hour over periods up to 14 days.

### MATERIALS AND METHOD

The temperature-raising apparatus is a modified thermostat. The principle, which was suggested by Dr R. H. J. Brown, is to draw the pin of a mercury-toluene thermostat away from the surface of the mercury at a known constant rate. The rate of rise of temperature then depends on the change in length of the mercury column in the thermostat capillary for  $1^{\circ}$  C. change in temperature and on the speed at which the cord is wound up. The cord must, therefore, be wound round a drum of calculated diameter which revolves at a fixed speed. By varying the diameter of the drum or the speed of revolution, any rate of rise can be obtained providing that the bore of the thermostat capillary is constant. A constant speed of revolution was supplied by a Venner clock, and use of a long capillary made it possible to raise the temperature  $10^{\circ}$  C. without changing the thermostat.

The five rates of temperature rise used were  $1/20$ ,  $1/10$ ,  $4/10$ ,  $5/10$  and  $8/10^{\circ}$  C. an hour. The last is sixteen times faster than the first and this range was chosen to include at least one speed ( $1/20^{\circ}$  C. an hour) at which acclimatization ought to be possible and speeds at which acclimatization ought not to be possible. The tests at  $1/20$ ,  $1/10$  and  $8/10^{\circ}$  C. an hour were each repeated four times. The  $4/10$  and  $5/10^{\circ}$  C. tests were not repeated as the results were not so interesting. The fish were

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used in samples of eight, except for one  $8/10^{\circ}\text{C}$ . test when only six fish were used and for the  $5/10^{\circ}\text{C}$ . test when only five fish were used. All the roach came from Tarn Hows (see Cocking, 1959) except for thirteen fish used for rates of  $4/10$  and  $5/10^{\circ}\text{C}$ . an hour which came from East Anglia.

The fish were acclimatized to  $20^{\circ}\text{C}$ . because it was the lowest temperature that could be maintained all the year round. The fish were starved for 48 hr. in the acclimatization tanks before being transferred and they were starved throughout the experiments to prevent the estimations of oxygen and ammonia being affected by decaying food and faeces.

The experiments were done in three 50 l. tanks in the tank room and environmental conditions were, therefore, carefully controlled (Cocking, 195 ). It was not possible to control accurately the volume of air bubbling through the tanks, but the volume per minute was set roughly equal in the control and experimental tanks at the beginning of the experiment.

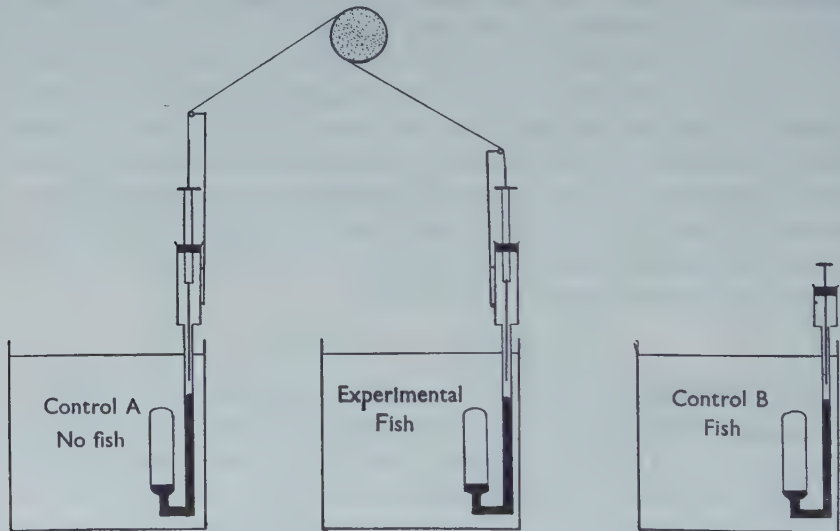


Fig. 1. Diagram of the apparatus used to investigate the effects on roach of temperatures rising continuously at known, constant rates. Control A and experimental tank—temperature rises at known constant rate. Control B—temperature constant at  $20^{\circ}\text{C}$ . throughout.

The experimental tank (Fig. 1) contained fish which were subjected to a known rate of temperature rise. Control A served as a check on the oxygen and ammonia concentrations of tap water containing no fish but undergoing the same rate of temperature rise as the experimental tank. Control B contained the same number of fish as the experimental tank, but they were maintained throughout at the acclimatization temperature ( $20^{\circ}\text{C}$ .) and this served as a control for the effects on oxygen and ammonia concentrations of starving fish at  $20^{\circ}\text{C}$ . Control B had a standard pattern thermostat set at  $20^{\circ}\text{C}$ ., while Control A and the experimental tank both contained long-necked thermostats; one clock raised the pins of both thermostats, so the rates of temperature rise in the two tanks were the same.

The three tanks were set at 20° C. and the fish were caught and put into Control B and the experimental tank. They were left for several hours to recover from the shock of transfer and the temperature-raising apparatus was then switched on, either by hand or by a Venner time switch. The temperature continued to rise until all the fish were dead and the time and temperature at which each fish died were noted. The dead fish were weighed and measured, sexed (where possible) and examined for parasites. Notes were made on the general condition of the fish, on the colour and size of the gall bladder and on whether the heart was still beating. Throughout the experiments notes were made on the behaviour of the fish. The oxygen and ammonia concentrations were estimated every 4 hr. from 10 a.m. to 10 p.m., but estimations were not made during the night except when the fish were about to die or were dying.

Chemical and physical estimations were made by the methods described by Cocking (1959).

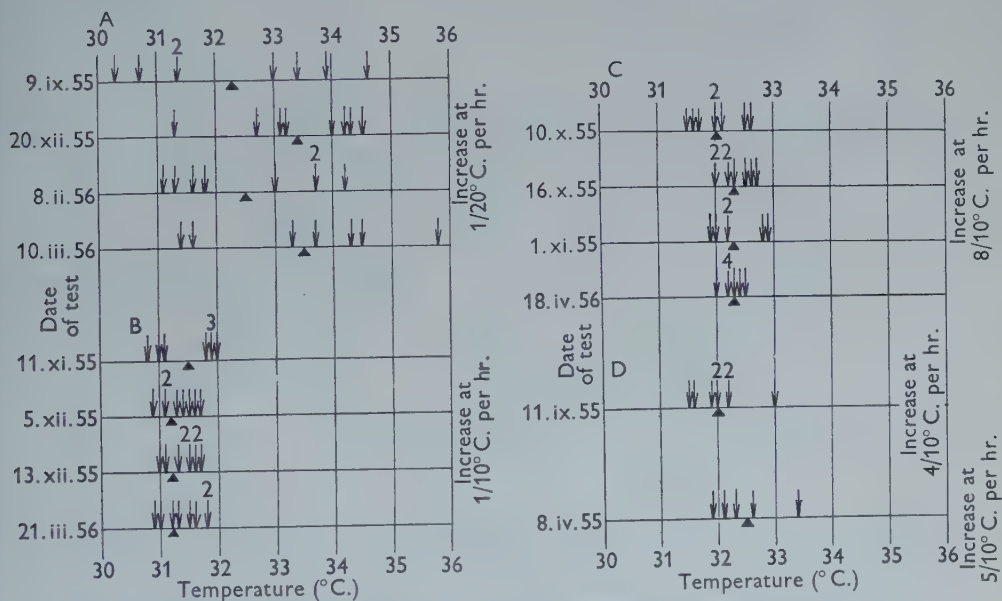


Fig. 2. Diagram showing the temperatures at which individual fish died when the water temperature was raised at constant rates. Each horizontal line represents a single test and each arrow marks the temperature at which a fish died. A figure above an arrow means that more than one fish died at that temperature. A solid black triangle marks the mean death temperature for each test. Vertical lines—temperatures in °C. Figures to the left of the lines—dates of the tests. A—temperature increased at 1/20° C. an hour; B—at 1/10° C. an hour; C—at 8/10° C. an hour; D—at 4/10 and 5/10° C. an hour.

## RESULTS

### *Temperatures and times of death*

Figure 2 shows the temperatures at which individual fish died and the mean for each test when the temperature was raised at 1/20, 1/10, 4/10, 5/10 and 8/10° C. an hour. The three most interesting results are the 1/20, 1/10 and 8/10° C. since these

have been investigated most fully, but the single trial tests at  $4/10$  and  $5/10^{\circ}$  C. an hour have been included for comparison.

Figure 3 compares the range over which the fish died for each rate of increase and there is a striking difference between the  $1/20^{\circ}$  C. result with a total range of  $5.5^{\circ}$  C. (mean  $32.9^{\circ}$  C.) and the  $1/10^{\circ}$  C. result with a range of only  $1.2^{\circ}$  C. (mean  $31.4^{\circ}$  C.). This difference is reflected in the times at which the fish died (Table 1). All the fish in any test with  $1/10^{\circ}$  C. an hour rise died in a period of 7–12 hr. and at  $8/10$ ,  $5/10$  and  $4/10^{\circ}$  C. an hour rise the fish died in roughly one-eighth, one-fifth and one-quarter of the time taken at  $1/10^{\circ}$  C. an hour rise. At  $1/20^{\circ}$  C. an hour rise, however, the fish took up to 88 hr. to die in a single test.

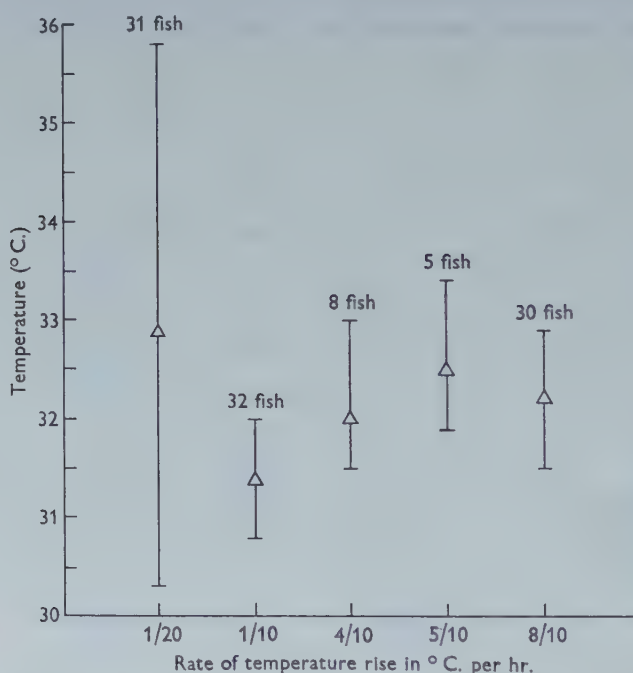


Fig. 3. Diagram comparing the total ranges over which the roach died when the temperature rose at  $1/20$ ,  $1/10$ ,  $4/10$ ,  $5/10$  and  $8/10^{\circ}$  C. an hour. The open triangle marks the mean death temperature for all fish tested at each rate. The figures above the lines are the total numbers of fish tested at that rate.

*The oxygen, free ammonia and carbon dioxide concentrations  
when the fish were dying*

The minimum concentration of dissolved oxygen recorded when the fish were dying was 5.1 p.p.m. in the  $8/10^{\circ}$  C. test on 10 October 1955; in all other tests where the concentration was estimated the minimum concentration when the fish were dying was 5.6 p.p.m.

The maximum concentration of free ammonia recorded when the fish were dying was 0.07 p.p.m. and the maximum free carbon dioxide concentration 4 p.p.m.

In view of the work of Wurhmann (1952) and Cocking (1959), it is unlikely that either low oxygen or high free ammonia was a limiting factor in the survival of the fish.

Table 1. *Comparison of the times of death of roach when the water temperature was raised at different rates*

Rate of increase ° C. an hour	Time first fish died in hours from start	Time last fish died in hours from start	Difference in hours
1/20	206-228	284-316	88-62
1/10	108-110	117-120	12-7
4/10	29	32½	3½
5/10	22	25	3
8/10	14½-15	15½-16	¾-1½

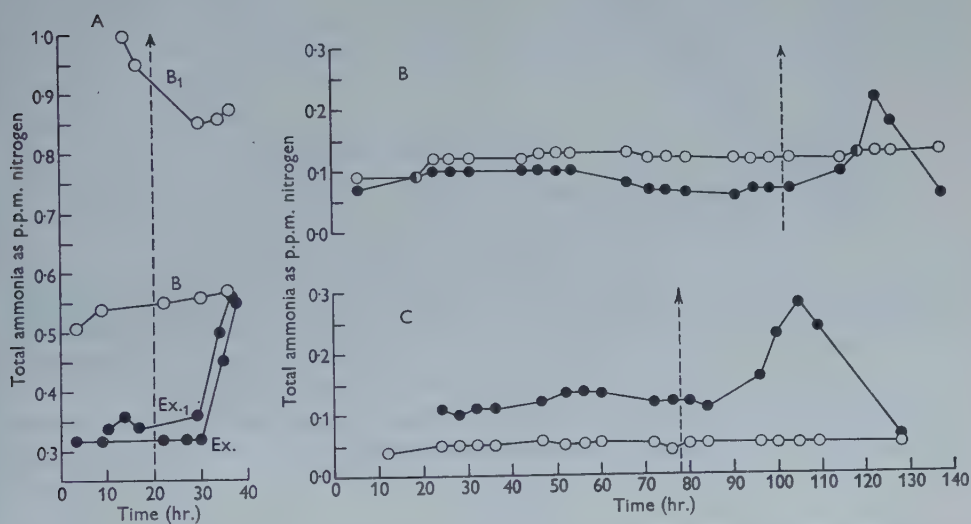


Fig. 4. Changes in the concentration of ammonia in experimental tanks with rising temperature and control tanks at 20° C. The vertical broken arrow marks the time at which the temperature began to rise. The fish were put into the tanks at 20° C. at zero hours. Solid circles—experimental fish; open circles—control fish. Rate of temperature rise—A, 8/10° C. an hour (Ex. and B—first test; Ex.<sub>1</sub> and B<sub>1</sub>—second test); B—5/10° C. an hour; C—4/10° C. an hour.

#### *Changes in the ammonia concentrations in the tanks*

Figures 4 and 5 show the change in the ammonia concentrations in five of the tests with different rates of temperature increase. In each, the temperature remained constant for several hours before the increase in temperature began. This period at 20° C. is shown in Fig. 4 but omitted from Fig. 5.

Control A (no fish) never contained more than a trace of ammonia, so all the ammonia measured in Control B and in the experimental tank came from the fish. The concentration of ammonia remained fairly constant throughout in Control B, though in one of the 8/10° C. tests (B<sub>1</sub> in Fig. 4A) it started high and fell rapidly during the test. In the experimental tanks it remained constant while the tem-

perature remained at  $20^{\circ}\text{C}$ . When the temperature started to rise the concentration did not start to rise immediately and the latent period was about 7 hr. in the  $8/10^{\circ}\text{C}$ . tests (equivalent to  $5.6^{\circ}\text{C}$ .), 11 hr. in the  $5/10^{\circ}\text{C}$ . tests (equivalent to a rise of  $5.5^{\circ}\text{C}$ .), 14 hr. in the  $4/10^{\circ}\text{C}$ . tests (equivalent to  $5.6^{\circ}\text{C}$ .) and about 50 hr. in the  $1/10^{\circ}\text{C}$ . test (equivalent to  $5.0^{\circ}\text{C}$ .). In the  $1/20^{\circ}\text{C}$ . tests, however, the latent period lasted for at least 140 hr. (equivalent to  $7.0^{\circ}\text{C}$ . rise in temperature) and in no case did the concentration rise steeply until after 240 hr. (tank temperature  $32^{\circ}\text{C}$ .). In all tests at rates faster than  $1/20^{\circ}\text{C}$ . an hour, the concentration was at a maximum at  $32^{\circ}\text{C}$ .

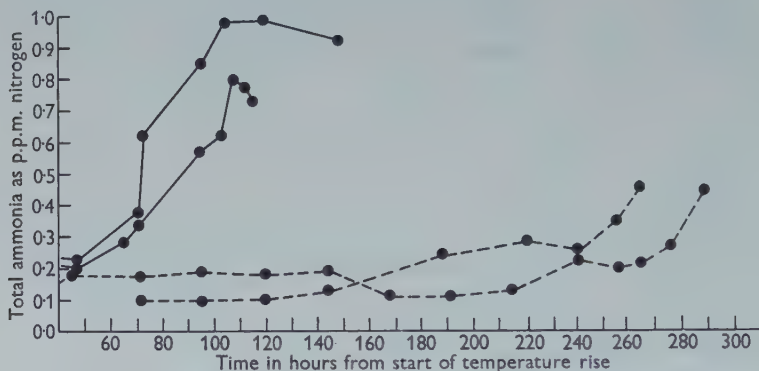


Fig. 5. Changes in the concentration of ammonia in the experimental tank when the temperature rises at  $1/10$  and  $1/20^{\circ}\text{C}$ . an hour. Temperature starts to rise at zero hours. Controls not shown. Solid lines—rate of rise of  $1/10^{\circ}\text{C}$ . an hour; broken lines—rate of rise of  $1/20^{\circ}\text{C}$ . an hour.

Roach, therefore, excrete more ammonia at high temperatures and the excretion is affected by the rate at which the temperature rises.

### Behaviour

The behaviour of roach in constantly changing temperatures can be described as a variation of the sequence of behaviour in constant lethal temperatures (Cocking 1959).

The behaviour series was similar at all rates though the stages took longer at the slower rates. The fish remained normal up to about  $31^{\circ}\text{C}$ . and then passed into stages of final distress, loss of control and death which were similar to those stages in the constant temperature tests. The beginning of final distress was marked by increased activity resulting in the breaking up of the shoal; in one  $1/20^{\circ}\text{C}$ . test, the fish became more active at  $30.9^{\circ}\text{C}$ . while in an  $8/10^{\circ}\text{C}$ . test activity increased at  $30.7^{\circ}\text{C}$ . Other characteristic signs of distress were darkening, very rapid shallow breathing (up to 250 movements a minute), inability to maintain position in the water and passing bubbles at the surface.

The behaviour differed, however, from that in the constant temperature tests in several ways. In experiments at three rates ( $1/20$ ,  $1/10$  and  $4/10^{\circ}\text{C}$ . an hour) one fish, usually the largest, became aggressive and appeared to keep a territory for itself

at the back of the tank and drive the other fish away, butting them behind the anal fin with its snout. Such behaviour was not seen in normal roach which always formed a shoal. Aggressive behaviour was first noted in the  $1/20^{\circ}$  test at  $30.5^{\circ}$  C., in the  $1/10^{\circ}$  test at  $28.2^{\circ}$  C. and in the  $4/10^{\circ}$  test at  $29.7^{\circ}$  C. It was, therefore, characteristic of a predistress stage, appearing well below the temperature at which the fish became distressed and disappearing again as the temperature rose higher.

The distressed fish did not show the characteristic dark pattern, though they invariably darkened. In the  $8/10$ ,  $5/10$ ,  $4/10$  and  $1/10^{\circ}$  C. tests the darkest, most obviously distressed fish usually died first, but this did not always apply in the  $1/20^{\circ}$  C. tests. In one  $1/20^{\circ}$  C. test, one easily distinguishable fish was very dark and at the surface of the water at  $30.9^{\circ}$  C.; by  $31.2^{\circ}$  C. this fish was making half-rolls at the surface but at  $32.2^{\circ}$  C. it was still alive and at the bottom of the tank, though three smaller fish, which had shown no signs of distress at  $31.2^{\circ}$  C. had by this time died. The large fish did not die until the water temperature reached  $33.8^{\circ}$  C., i.e. 60 hr. after the first signs of distress. Temporary recovery of fish after showing distress was characteristic of the  $1/20^{\circ}$  C. tests and was noted on several occasions.

The fish died quickly once they had lost control. The time between rolling over (loss of control) and death in eight  $8/10^{\circ}$  C. fish was 4, 4, 3, 2, 3, 4, 2 and 3 min., respectively, and even at slower rates of temperature increase death usually occurred a few minutes after rolling over. As the fish died, the myotomic muscles were the first to stop working and the mouth and opercular muscles the last. The general sequence was, therefore, a long period of distress marked by darkening and greater activity, followed by loss of control and rapid death, with the head muscles being the last to stop working.

When the body cavity was opened after death, the heart was still beating in most cases and in fish that had undergone a long exposure to high temperatures (i.e. a temperature increasing at  $1/20^{\circ}$  C. an hour), the gall bladder was characteristically black and large.

#### DISCUSSION

The rate of temperature rise affects both the time the fish have for acclimatization and the time they are exposed to lethal temperatures. If the temperature rises sufficiently slowly, the fish can acclimatize fully, while at the other extreme, the temperature may rise so quickly that no acclimatization is possible. If there is no acclimatization, the faster the rise in temperature, the higher will be the death temperature as Jacobs (1919) demonstrated with starfish larvae. When, however, the temperature rises at a rate that is intermediate between the rate for complete acclimatization and no acclimatization, the death temperature will depend on the interaction between the length of exposure at lethal temperatures and the chance to acclimatize at the given rate. For all rates faster than the rate for complete acclimatization, therefore, the beneficial effect of a slow rate (more chance to acclimatize) is tempered by the harmful effects of longer exposure in the lethal zone.

In all these tests, the roach were acclimatized to  $20^{\circ}$  C. The temperature for instantaneous death at this acclimatization temperature is  $31.5^{\circ}$  C. (Cocking, 1959).

The mean temperature for the death of all the  $1/20^{\circ}$  C. fish ( $32.9^{\circ}$  C., Fig. 3) was the highest for any rate of increase and in two tests the means (Fig. 2) coincided with the ultimate upper lethal temperature for the species ( $33.5^{\circ}$  C.). The time taken for the fish to die (Table 1) and the temperature range over which the fish died (Fig. 3) were also disproportionately long when compared with other rates. A rate of  $1/20^{\circ}$  C. an hour rise in temperature ( $1.2^{\circ}$  C. a day), therefore, allows complete acclimatization to take place in roach. Fry, Hart & Walker (1946) acclimatized *Salvelinus fontinalis* at a rate of  $1^{\circ}$  C. rise in temperature a day and it is possible that all fish can acclimatize fully at this rate.

At  $1/10^{\circ}$  C. an hour rise, the fish died over the lowest range for all rates used; presumably acclimatization was incomplete and the exposure in the zone of resistance to lethal temperatures must be at a maximum. In the  $1/10^{\circ}$  C. tests the first fish died at about  $30.8^{\circ}$  C. but not until  $31.5^{\circ}$  C. in the  $8/10^{\circ}$  C. tests. This is because the lethal experience of the  $8/10^{\circ}$  C. fish, i.e. the length of time spent in the zone of resistance above  $29^{\circ}$  C. is only one-eighth of that of the  $1/10^{\circ}$  C. fish. Some acclimatization must have occurred in the  $8/10^{\circ}$  C. fish, since they did not start to die until the temperature for instantaneous death was reached ( $31.5^{\circ}$  C.) and most died above that temperature. Partial acclimatization in the roach must, therefore, be rapid and this agrees with what is known about the speed of acclimatization in other species (Loeb & Wasteneys, 1912).

The  $5/10^{\circ}$  C. test (Fig. 3) gave the highest mean and range for any speed of temperature rise faster than the rate for complete acclimatization, while the  $4/10^{\circ}$  C. test had about the same range and a slightly lower mean than the  $8/10^{\circ}$  C. tests. It is possible, therefore, that  $5/10^{\circ}$  C. an hour rise represents an optimum interaction between the time allowed for acclimatization and the harmful effects of exposure to lethal temperatures. The  $4/10$  and  $5/10^{\circ}$  C. results are not, however, so well established as the results at the other rates and the fish used were from a different stock, i.e. from East Anglia instead of from Furness.

Since the fish were starved throughout the tests, ammonia must have been produced at the expense of body proteins and protein catabolism must have been greater as the death temperature was approached. Roach kept at high, sublethal temperatures lose weight, even when fed, and show pronounced muscular wasting (Cocking, 1957) and Rasquin & Rosenbloom (1954) found that eyed *Astyanax* catabolized protein during stress induced by darkness, though fat deposits increased in the tissues. It is probable, therefore, that the increase in ammonia excretion by the roach is a result of protein catabolism induced by heat stress. The disproportionately long latent period when the temperature rose at  $1/20^{\circ}$  C. an hour (Fig. 5), thereby allowing complete acclimatization, further suggests that a high rate of excretion may be associated with stress during incomplete acclimatization to high temperatures.

The fish died from the posterior end forwards, the myotomic muscles passing into rigor first and the mouth and opercular muscles last. A similar posterior-anterior gradient of death was noted in roach dying at high constant lethal temperatures (Cocking, 1959). The causes of the death of fish at high temperatures are

not properly understood. Gibson (1954) deduced from statistical data that heat death was complex in *Lebistes*. Brett (1952) found evidence for three factors in death from cold in *Oncorhynchus* and Fry (personal communication) does not think that the cause of death is a single factor. The hearts were beating when the roach were opened, both in the present tests and in those at constant temperatures, though the beat was often feeble. This observation does not agree with the work of Battle (1926) or Vernon (1899), who found that the heart muscle was more susceptible to heat than skeletal muscle. An abnormal heart beat, however, would cause poor circulation and this would rapidly cause anoxia at high temperatures when the oxygen consumption of the tissues must be at a maximum. Such an imbalance between the oxygen supply to the tissues and the oxygen required by them would rapidly lead to death.

## SUMMARY

1. When roach were acclimatized to 20° C. and then subjected to five constant rates of temperature rise, the range of death temperatures depended on the interaction between the opportunity for acclimatization and the exposure to lethal temperatures.

2. At 1/20° C. an hour rise in temperature, roach acclimatized fully, died over the longest temperature range (30.3–35.8° C.) had the highest mean death temperature (32.9° C.) and died over a disproportionate length of time (up to 88 hr.) when compared with other rates. The death temperature range at 1/10° C. an hour was 30.8–32.0° C. (mean 31.4° C.) and at 8/10° C. an hour was 31.5 to 32.9° C. (mean 32.9° C.).

3. Roach cannot acclimatize fully at rates faster than 1/20° C. an hour though some acclimatization takes place at a rate of 8/10° C. an hour.

4. Roach excrete more ammonia as the temperature rises but the increase depends on the rate of temperature rise and is delayed for up to 240 hr. at 1/20° C. an hour.

5. The behaviour showed characteristic changes.

6. Roach died from the posterior end forwards; the heart was beating and the gall bladder abnormal when the body was opened.

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Charts and curves can often be drawn to best advantage on *graph paper ruled in pale blue.* The blue lines, while ensuring accuracy, are easily eliminated by the printer, only the blackened lines that are desired remaining.

(ii) *Text half-tone blocks.* These are suitable for illustrations involving brushwork, or in which the depth of shading is an essential feature. They can be used for such things as oscillograph records and some photographs can be produced in this way, a good glossy bromide print being required. Illustrations should be gummed on *white card*, grouped and numbered as they are to appear in print. All lettering should be shown in position on a covering sheet of transparent paper.

(iii) *Plates.* Plates should be used only for illustrations, such as photomicrographs, in which the most accurate reproduction of fine detail is called for. Plates are expensive and the Editors may require an author to defray the cost of plates which in their opinion are not essential. The photographs making up the plate should be gummed on *white card*, grouped and numbered as they are to appear in print. Exclusive of margin the plate figures should not cover, when reduced, an area greater than  $7\frac{1}{2}$  in. in length  $\times$  5 in. in width when ready for reproduction as a single plate, or  $7\frac{1}{2}$  in.  $\times$   $11\frac{1}{2}$  in. in the case of double plates. All lettering should be shown in position on a covering sheet of transparent paper.

Authors are asked not to submit sheets of illustrations which are more than foolscap size; or, if this cannot be avoided, to include photographic reductions for the convenience of referees.

#### ABSTRACTS:

Authors should submit with their MSS. *four copies* (typewritten, double spacing) of an abstract suitable for biological abstracting journals. The abstract will not appear in the *Journal of Experimental Biology* but will be scrutinised by the Editors before being passed for publication. The summary of a paper may serve as an abstract provided that it conforms to the following requirements. The abstract should outline as briefly as possible the results and the definitive conclusions of the work. Details of methods are generally not required. A paper of average length should be abstracted in about 100 words and the abstract should never exceed 3 % of the original. An address (to which applications for offprints may be sent) should be added.

#### PROOFSHEETS AND OFFPRINTS:

Authors will receive one set of slip proofs for correction and return to the Editors. A page proof will also be sent if the slip proof is marked by the author 'Revise'. An allowance of ten shillings per sheet of sixteen pages will be made for alterations apart from printer's errors. Authors may be charged for any excess over this average. Authors will receive 50 copies of their papers free; additional copies may be purchased and should be ordered when the proofs are returned to the Editors.

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## THE SOCIETY FOR EXPERIMENTAL BIOLOGY

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